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(54) Title: DIAGNOSTICS AND VACCINES FOR MYCOBACTERIAL INFECTIONS OF ANIMALS AND HUMANS

(57) Abstract

This invention relates to the protein, *Mycobacterium paratuberculosis* acylase (*mpa*) and the gene encoding *mpa*, which we have identified in the pathogen *Mycobacterium paratuberculosis* *Mptb* (also designated *Mycobacterium avium* subspecies *paratuberculosis MAP*), and to their use in the diagnosis of *Mptb/MAP* infections in animals and humans, as well as their use as components of vaccines for the prevention and treatment of diseases caused by *Mptb/MAP*. The importance of an intact uninterrupted *mpa* gene as a determinant of pathogenicity in *Mptb/MAP* is recognised and the invention also provides attenuated strains of normally pathogenic *Mptb/MAP* and other mycobacteria in which *mpa* has been inactivated, for use as vaccines.

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**DIAGNOSTICS AND VACCINES FOR MYCOBACTERIAL INFECTIONS OF ANIMALS AND HUMANS.**

This invention relates to the protein, *Mycobacterium paratuberculosis* acylase (*mpa*) and  
5 the gene encoding *mpa*, which we have identified in the pathogen *Mycobacterium paratuberculosis Mptb* (also designated *Mycobacterium avium* subspecies *paratuberculosis MAP*), and to their use in the diagnosis of *Mptb/MAP* infections in animals and humans, as well as their use as components of vaccines for the prevention and treatment of diseases caused by *Mptb/MAP*. We recognise the importance of an intact uninterrupted *mpa* gene as  
10 a determinant of pathogenicity in *Mptb/MAP*. Thus the invention also provides attenuated strains of normally pathogenic *Mptb/MAP* and other mycobacteria in which *mpa* has been inactivated, for use as vaccines. In the remainder of this specification the abbreviation *MAP* will be used to denote the pathogen *Mycobacterium paratuberculosis* (*Mycobacterium avium* subspecies *paratuberculosis*). The skilled person would clearly appreciate that the  
15 abbreviations *MAP* and *Mptb* can be used interchangeably.

*MAP* is a pathogenic mycobacterium and a member of the group of mycobacteria called *M.avium-intracellulare* (MAIC). *MAP* causes Johne's disease, a chronic inflammation of the intestine of a broad range of different types in many species of animals including  
20 primates. *MAP* also causes Crohn's disease in humans and other chronic inflammatory diseases such as sarcoidosis. Johne's disease is widespread in Europe and North America as well as elsewhere, and is a major problem in both domestic and wild animals causing substantial economic losses. Crohn's disease in humans is increasing in frequency in Western Europe, North America and elsewhere, and is a major cost to health services.  
25

The diagnosis, prevention and treatment of *MAP* infections present major problems for veterinary and human medicine, as well as for public health. The development of effective new diagnostics and vaccines for the recognition, prevention and/or treatment of *MAP* disease, depend upon identifying specific genes and their products within *MAP* that are  
30 both highly specific to *MAP* and associated with pathogenicity. *MAP* is very similar to

other non-pathogenic members of the MAIC, but some of its genes such as IS900 and the genes of the GS element, are known to be specific or highly selective for this organism and are associated with pathogenicity. Thus, IS900 and GS element polynucleotides and polypeptides are useful in diagnosis and as components of vaccines against *MAP*.

5

We have now identified a new gene which occurs naturally within the genome of *MAP* and which encodes a new protein, *mpa*. This is termed the *mpa* gene of the invention. The nucleotide sequence of the *mpa* gene is given in SEQ ID No. 1 and the amino acid sequence of the *mpa* polypeptide it encodes is given in SEQ ID No. 2. The uninterrupted 10 *mpa* gene is specific for *MAP*. Unlike the genes in the GS element, homologues of the *mpa* gene are not present in pathogenic *Mycobacterium tuberculosis*. Furthermore, we have found that the equivalent gene to *mpa* in the closely related but less pathogenic *M.avium* subsp. *silvaticum* (*Mavs*) and *M.avium* TMC724 ATCC 25291 is interrupted by the presence of an IS21-like insertion sequence, IS1612. The nucleotide sequence of IS1612 is 15 given in SEQ ID No. 3 and its complement in SEQ ID No. 4. Nucleotides 1856-2543 of SEQ ID No. 3 and nucleotides 1-688 of SEQ ID No. 4 are new and constitute further aspects of the invention. All or part of the polynucleotide sequence of IS1612 may be used to deactivate *mpa* in *MAP*.

- 20 We have found that the *mpa* gene encodes a polypeptide (SEQ ID No.2) whose function is to acylate or acetylate cell wall components, particularly fucose sugars, in *MAP*. More precisely *mpa* functions in conjunction with the five polypeptides encoded by the ORFs present in the GS region of *MAP*. The five polypeptides encoded by the GS are: *gsa* a sugar transferase, *gsbA* and *gsbB* which function in tandem to produce fucose, *gsc* a methylase, 25 and *gsd* a fucosyl transferase. These five polypeptides of GS serve to provide glycosyl peptidolipids (GPL) which comprise a fucosyl moiety and which are located in the mycobacterial cell wall. The methylase *gsc* acts to methylate, among other groups, the fucosyl moiety of the GPL making it less recognisable by the host's immune system. The acylase, *mpa*, acts as an acetylating agent on, among other groups, the fucosyl moiety. 30 Acetylation of this GPL broadens the range of animals and host cells which can be infected

by *MAP*.

Acetylation of surface polysaccharides is important in host cell recognition. For example, it is known that de-acetylation of the terminal fucose of GPL's from *M.avium* MAC serotype 5 9 abolishes rabbit anti-serotype 9 antibody agglutination indicating that acetylation plays a key role in strain variability amongst bacteria and may be directly attributable as a virulence factor. Acetylation of rhamnose in GPL's of *Mycobacterium smegmatis* confers resistance to mycobacteriophage D4 by inducing conformational changes that destroy the phage attachment site. In *MAP*, the *mpa* gene is responsible for modification of terminal 10 sugar residues in *MAP* GPL's which are critically important in determining cell surface recognition and receptor binding and are important determinants of pathogenicity.

Acetylation of LPS's is also known to be critical in destroying antibody epitopes in *S.typhimurium* (Slauch J.M., Lee A.A., Mahan M.J., Mekalanos J.J., J.Bacteriol. 1996, 15 178, 5904-5909).

Furthermore, acetylation of LPS's is known to increase the virulence of bacteria such as *S.flexneri* (Clark C.A., Beltrame J. and Manning P.A., Gene 1991, 107, 43-52).

20 Due to its specificity to *MAP* and some pathogenic *M.avium* ser2, and the involvement of *mpa* in the biosynthesis and modification of cell wall components, *mpa* polypeptide can be used as a target for immunodiagnostic tests for the recognition of *MAP* infections in animals and humans. Furthermore, immunisation of animals or humans with *mpa*-derived products such as naked *mpa* DNA in suitable expression constructs, or *mpa* polypeptide or 25 fragments thereof with adjuvants and carriers, will enhance the immunological resistance of animals and humans to *MAP* infections. Specific fragments of *mpa* polypeptide which are particularly suitable targets for diagnostic tests and peptide vaccines can be selected by structural analysis using computer programs well known in the art

It is also an objective of the present invention to provide new methods for diagnosing Johnne's disease in animals and Crohn's disease in humans using immunoassays based on antibody and cell mediated immune reactivity to *mpa* or fragments thereof. It is a further objective of the present invention to prevent and/or treat *MAP* infections in animals and

5      humans by vaccination using the polynucleotides or polypeptides of the *mpa* gene either alone or together with other polynucleotides and polypeptides associated with pathogenicity, such as those of GS and IS900 known in the art. We recognise that vaccination using naked *mpa* DNA together with naked GS DNA both in suitable expression constructs will result in the provision of GPL structures on the cell surface of

10     suitable recipient cells, which GPL will resemble those of *MAP* resulting in the generation of protective immunity. We further recognise that attenuated mutants of *MAP* provided by interruption or deletion of the *mpa* gene will be suitable for use as vaccines.

Accordingly, the present invention provides a polynucleotide encoding a protein that

15     comprises mycobacterium paratuberculosis acylase (*mpa*), or a fragment or homologue thereof having *mpa* activity.

The invention also provides a polynucleotide selected from:

- (a)    a polynucleotide comprising the nucleotide sequence set out in SEQ ID No. 1 or the complement thereof;
- 20     (b)    a polynucleotide comprising a nucleotide sequence capable of hybridising to a fragment of the nucleotide sequence set out in SEQ ID No. 1, the fragment having the nucleotide sequence of nucleotides 210-1335 of SEQ ID No. 1;
- 25     (c)    a polynucleotide comprising a nucleotide sequence capable of hybridising to the complement of a fragment of the nucleotide sequence set out in SEQ ID No. 1, the fragment having the nucleotide sequence of nucleotides 210-1335 of SEQ ID No. 1;
- 30     (d)    a polynucleotide comprising a polynucleotide sequence which is degenerate as a result of the genetic code to the polynucleotide of SEQ ID No. 1 or a polynucleotide of (c); and

- (e) a polynucleotide having at least 80% homology to the nucleotide sequence of SEQ ID No. 1.

The polynucleotide preferably encodes a polypeptide having *mpa* activity.

- 5 The invention also provides a polynucleotide probe or primer which comprises a fragment of at least 15 nucleotides of a polynucleotide selected from:
- (b) a polynucleotide comprising a nucleotide sequence capable of hybridising to a fragment of the nucleotide sequence set out in SEQ ID No. 1, the fragment having the nucleotide sequence of nucleotides 210-1335 of SEQ ID No. 1;
- 10 (c) a polynucleotide comprising a nucleotide sequence capable of hybridising to the complement of a fragment of the nucleotide sequence set out in SEQ ID No. 1, the fragment having the nucleotide sequence of nucleotides 210-1335 of SEQ ID No. 1; and
- (d') a polynucleotide comprising a polynucleotide sequence which is degenerate as a  
15 result of the genetic code to a polynucleotide sequence of (c).

The invention also provides a polypeptide encoded by a polynucleotide of the invention, which is not in its natural environment and is preferably in substantially isolated form.

- 20 The invention also provides a polypeptide which is not in its natural environment and is preferably in substantially isolated form, which comprises the sequence set out in SEQ ID No. 2, or a polypeptide substantially homologous thereto which has *mpa* activity, or a fragment of the polypeptide of SEQ ID No. 2 which has *mpa* activity. The invention also provides a polypeptide comprising at least 8 amino acids which is an immunogenic  
25 fragment of said polypeptides and which comprises an epitope. Preferred polypeptides comprise amino acid residues 158-211 and 380-444 of SEQ ID No. 2.

The invention also provides a vector comprising a polynucleotide of the invention.

- 30 Preferably the vector is an expression vector comprising a polynucleotide of the invention

operably linked to regulatory sequences capable of directing expression of said polynucleotide in a host cell. The invention also provides an antibody capable of recognising a polypeptide of the invention including preferred polypeptides. Preferably an antibody which is a monoclonal antibody or a fragment thereof.

5

The invention also provides a method for detecting the presence or absence of a polynucleotide of the invention in a biological sample which method comprises:

- (a) bringing a biological sample containing DNA or RNA into contact with a probe of the invention under hybridising conditions; and
- 10 (b) detecting any duplex formed between the probe and nucleic acid in the sample.

The invention also provides a method of detecting the presence or absence of a polypeptide of the invention in a biological sample which method comprises:

- (a) incubating the biological sample with an antibody of the invention under conditions 15 which allow for the formation of an antibody-antigen complex; and
  - (b) determining whether antibody-antigen complex comprising said antibody is formed.
- In such a method a substance which is capable of binding the polypeptide of the invention in a specific manner could be used instead of the antibody, and thus (b) would comprise determining whether a complex was formed between the substance and the polypeptide of 20 the invention.

The invention also provides a method of detecting the presence or absence of antibodies to a polypeptide of the invention in a biological sample which method comprises:

- (a) incubating a biological sample with a polypeptide of the invention comprising an 25 epitope under conditions which allow for the formation of an antibody-antigen complex; and
- (b) determining whether an antibody-antigen complex comprising said polypeptide is formed.

30 The invention also provides use of a polypeptide of the invention to detect the presence or

absence of cell-mediated immune reactivity in an animal or human to the polypeptide.

Typically such a method comprises contacting the polypeptide with an immune cell from the human or animal in order to determine the presence or absence of a cellular immune response. In particular the invention provides a method of detecting the presence or

5 absence of cell mediated immune reactivity in an animal or human, to a polypeptide of the invention which method comprises:

- (a) incubating a cell sample with a polypeptide of the invention comprising an epitope under conditions which allow for a cellular immune response; and
- (b) detecting the presence of said cellular immune response in the incubate.

10

The invention also provides a test kit for detecting the presence or absence of a pathogenic mycobacterium in a sample which comprises a polynucleotide, a polypeptide or an antibody of the invention.

15 The invention also provides a pharmaceutical composition comprising (i) a polypeptide, a polynucleotide or an antibody of the invention and (ii) a suitable carrier or diluent.

The invention also provides a polypeptide, a polynucleotide or an antibody of the invention for use in the treatment, prevention or diagnosis of a disease caused by a mycobacterium.

20

The invention also provides a method of treating or preventing a mycobacterial disease in an animal or human caused by mycobacteria which express a polypeptide of the invention which method comprises administering to the animal or human an effective amount of said polypeptide.

25

The invention also provides a method of treating or preventing a mycobacterial disease in animals or humans caused by mycobacteria containing the nucleotide sequence of SEQ ID No. 1, which method comprises administering to the animal or human an effective amount of a polynucleotide or a vector of the invention.

30

Preferably the methods of treating mycobacterial disease of the invention are used against Johnne's disease or Crohn's disease.

The invention also provides a method for increasing the *in vivo* susceptibility of  
5 mycobacteria to antimicrobial drugs.

The invention also provides a vaccine composition comprising (i) a polypeptide, a  
polynucleotide or a vector of the invention together with (ii) a pharmaceutically acceptable  
carrier or diluent.

10

The invention also provides a plasmid containing a polynucleotide sequence of the  
invention under the control of a promoter.

The invention also provides a nucleic acid vaccine comprising (i) a plasmid of the  
15 invention; and (ii) a pharmaceutically acceptable carrier or diluent. Preferred nucleic acid  
vaccines which further comprise a transfection agent.

The invention also provides a vaccine comprising (i) a polypeptide of the invention  
optionally linked to a hapten molecule, and (ii) a pharmaceutically acceptable carrier or  
20 diluent.

The invention also provides a non-pathogenic microorganism or a cell from a human or  
animal species prone to infection by *mpa*-containing mycobacteria comprising a  
component on its surface which has been modified by a polypeptide of the invention.

25

The invention also provides a non-pathogenic microorganism or a cell from a human or  
animal species prone to infection by *mpa*-containing mycobacteria which has been  
transformed or transfected with a nucleic acid construct comprising a polynucleotide or  
plasmid of the invention, preferably the nucleic acid construct further comprises a

polynucleotide which encodes the polypeptides of the GS region of *MAP*. Most preferably the gene or genes present in the nucleic acid construct are expressed.

The invention also provides a vaccine comprising (i) a non-pathogenic microorganism or a 5 cell from a human or animal of the invention and (ii) a pharmaceutically acceptable carrier or diluent.

The invention also provides a non-pathogenic microorganism or a cell from a human or animal species prone to infection by *mpa*-containing mycobacteria comprising on its 10 surface an antigenic determinant capable of being produced by the action of a polypeptide of the invention and which is capable of eliciting antibodies which bind the surface of *MAP*.

The invention also provides a normally pathogenic mycobacterium or pathogenic isolate 15 thereof, whose pathogenicity is mediated in all or in part by the presence or expression of a polypeptide of the invention, which mycobacterium or isolate harbours an attenuating mutation in the polynucleotide sequence of the invention.

The invention also provides a vaccine comprising the mycobacterium or isolate of the 20 invention and a pharmaceutically acceptable carrier or diluent. Preferably the attenuating mutation in the mycobacterium or isolate comprised in the vaccine is mediated by the insertion of one or more nucleotides.

The invention also provides a polynucleotide insertion element selected from:

- 25 (a) a polynucleotide comprising the nucleotide sequence set out in SEQ ID Nos. 3 or 4;  
(b) a polynucleotide comprising a nucleotide sequence capable of hybridising to a fragment of the nucleotide sequence set out in SEQ ID No. 3, the fragment having the nucleotide sequence of nucleotides 1856-2543 of SEQ ID No. 3;  
(c) a polynucleotide comprising a nucleotide sequence capable of hybridising to a fragment of the nucleotide sequence set out in SEQ ID No. 4, the fragment having  
30

- the nucleotide sequence of nucleotides 1-688 of SEQ ID No. 4;
- (d) a polynucleotide comprising a polynucleotide sequence which is degenerate as a result of the genetic code to the polynucleotide of SEQ ID No. 4 or a polynucleotide of (b);
- 5 (e) a polynucleotide having at least 75% homology to the nucleotide sequence of SEQ ID No. 3; and
- (f) a polynucleotide having at least 75% homology to the nucleotide sequence of SEQ ID No. 4.
- 10 The invention provides as a preferred embodiment a vaccine comprising a mycobacterium or isolate of the invention wherein the attenuating mutation is mediated by insertion of a polynucleotide insertion element of the invention.
- 15 The invention also provides a polynucleotide probe or primer which comprises a fragment of at least 15 nucleotides of an insertion element of the invention, optionally carrying a revealing label.
- The invention also provides a polypeptide in substantially isolated form which is encoded by an insertion element polynucleotide of the invention.
- 20 The invention also provides a polypeptide comprising at least 8 amino acids which is an immunogenic fragment of an insertion element polypeptide of the invention and which comprises an istA epitope.
- 25 The invention also provides a vector comprising an insertion element polynucleotide of the invention.
- The invention also provides an expression vector comprising an insertion element polynucleotide of the invention, operably linked to regulatory sequences capable of directing 30 expression of said polynucleotide in a host cell.

The invention also provides a method for preparing a mycobacterium or pathogenic isolate of the invention which method comprises transfecting animal or human isolate of an *mpa* containing pathogenic bacterium with a polynucleotide construct comprising an insertion element polynucleotide of the invention. In preferred embodiments transfection is effected by electroporation. In most preferred embodiments the polynucleotide comprised in the construct has the nucleotide sequence set out in SEQ ID No. 3 or 4.

Detailed description of the invention.

10

A. Polynucleotides.

Polynucleotides of the invention may comprise DNA or RNA. They may be single or double stranded. They may also be polynucleotides which include within them synthetic or 15 modified nucleotides. Polynucleotides of the invention include polynucleotides in substantially isolated and isolated form. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that 20 the polynucleotides described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of polynucleotides of the invention.

Polynucleotides of the invention include:

- 25 i. a polynucleotide comprising the nucleotide sequence set out in SEQ ID No. 1 or the complement thereof;
- ii. a polynucleotide comprising a polynucleotide sequence capable of hybridising to a polynucleotide having the sequence set out in SEQ ID No. 1 or a fragment thereof, preferred fragments having the sequence set out in nucleotides 110-1335 preferably  
30 210-1335 of SEQ ID No. 1;

- iii. a polynucleotide comprising a nucleotide sequence capable of hybridising to the complement of a polynucleotide having the sequence set out in SEQ ID No. 1 or a fragment thereof, preferred fragments having the nucleotide sequence set out in nucleotides 110-1335 preferably 210-1335 of SEQ ID No. 1;
- 5 iv. a polynucleotide comprising a polynucleotide sequence which is degenerate as a result of the genetic code to the nucleotide of SEQ ID No. 1 or a polynucleotide of (iii);
- v. a polynucleotide having at least 80% homology to the nucleotide sequence of SEQ ID No. 1 or its complement;
- 10 vi. a polynucleotide comprising a nucleotide sequence set out in SEQ ID No. 3 or the complement thereof (SEQ ID No. 4);
- vii. a polynucleotide comprising a nucleotide sequence capable of hybridising to a fragment of a polynucleotide having the sequence set out in SEQ ID No. 3, the fragment having the nucleotide sequence set out in nucleotides 1856-2543 of SEQ
- 15 ID No. 3;
- viii. a polynucleotide comprising a nucleotide sequence capable of hybridising to a fragment of a polynucleotide having the sequence set out in SEQ ID No. 4, the fragment having the nucleotide sequence of nucleotides 1-688 of SEQ ID No. 4;
- ix. a polynucleotide comprising a polynucleotide sequence which is degenerate as a
- 20 result of the genetic code to the polynucleotide of SEQ ID No. 4 or the polynucleotide of (vii);
- x. a polynucleotide having at least 75% homology to the nucleotide sequence of SEQ ID No. 3; and
- xi. a polynucleotide having at least 75% homology to the nucleotide sequence of SEQ
- 25 ID No. 4.

Polynucleotides of the invention which are described as capable of hybridising to all or part of the DNA of SEQ ID No. 1 or the complements thereof or fragments of SEQ ID Nos. 3 and 4 will be generally at least 70%, preferably at least 75%, 80% or 90% or more preferably at least 95% homologous to the DNA to which they are described as hybridising

over a region of at least 20, preferably at least 25 or 30 for instance at least 40, 60 or 100 or more contiguous nucleotides.

It is to be understood that skilled persons may, using routine techniques, make nucleotide  
5 substitutions that do not affect the polypeptide sequence encoded by the polynucleotides of  
the invention to reflect the codon usage of any particular host organism in which the  
polypeptides of the invention are to be expressed.

Any combination of the above mentioned degrees of homology and minimum sizes may be  
10 used to define polynucleotides of the invention, with the more stringent combinations (i.e.  
higher homology over longer lengths) being preferred. Thus for example a polynucleotide  
which is at least 80% homologous over 25, preferably 30 nucleotides forms one aspect of  
the invention, as does a polynucleotide which is at least 90% homologous over 40  
nucleotides.

15 The homologues typically hybridise with the relevant polynucleotide at a level  
significantly above background. The signal level generated by the interaction between the  
homologue and the polynucleotide is typically at least 10 fold, preferably at least 100 fold,  
as intense as 'background' hybridisation. The intensity of interaction may be measured,  
20 for example, by radiolabelling the probe, e.g. with  $^{32}\text{P}$ . Selective hybridisation is typically  
achieved using conditions of medium to high stringency (for example 0.03M sodium  
chloride and 0.03M sodium citrate at from about 50°C to about 60°C). Methods of  
measuring polynucleotide homology are well known in the art. For example, the  
BLAST/N, BLAST/P and BLAST/X algorithms, for example used on their default settings,  
25 can be used to line up the sequences (as described in Altschul S. F. (1993) J Mol Evol  
36:290-300; Altschul, S, F *et al* (1990) J Mol Biol 215:403-10) and to calculate the  
polynucleotide homologies discussed herein. These algorithms can also be used to  
calculate the polypeptide homologies referred to below.

The homologue may differ from a sequence in the relevant polynucleotide by at least 1, 2, 5, 10 or more substitutions, deletions or insertions over a region of over 25, preferably 30 nucleotides, for instance at least 40, 60 or 100 or more contiguous nucleotides of the homologue. Thus the homologue and polynucleotide may differ by 1, 2, 5, 10 , 30 or more 5 substitutions, deletions or insertions.

- Polynucleotides of the invention may be in a substantially isolated form. It will be understood that the polynucleotide may be mixed with carriers or diluents which will not interfere with the intended purpose of the polynucleotide and still be regarded as 10 substantially isolated. A polynucleotide of the invention may also be in a substantially purified form, in which case it will generally comprise the polynucleotide in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the polynucleotide in the preparation is a polynucleotide of the invention.
- 15 Polynucleotides of the invention may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments, such as fragments of the polynucleotides mentioned herein, will be at least 15, preferably at least 20, for 20 example at least 25, 30 or 40 nucleotides in length, and are also encompassed by the term polynucleotides of the invention.

Polynucleotides such as a DNA polynucleotide and primers according to the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the 25 art. They may also be cloned by standard techniques.

In general, primers will be produced by synthetic means, involving a step wise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

Longer polynucleotides will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of about 15-30 nucleotides) to a region of the *mpa* gene which it is desired to clone, bringing the primers into contact with mRNA or cDNA, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector. Although in general the techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (1989) and Ausubel *et al.*, Current Protocols in Molecular Biology (1995), John Wiley & Sons, Inc.

Polynucleotides which are not 100% homologous to the sequences of the present invention but fall within the scope of the invention can be obtained in a number of ways. Other isolates or strains of pathogenic mycobacteria will be expected to contain allelic variants of the *mpa* sequence described herein may be obtained for example by probing genomic DNA libraries made from such pathogenic mycobacteria. In addition, other mycobacterial homologues of *mpa* in pathogenic *M. avium* such as *M. avium* strain 104 may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridising to SEQ ID No. 1. Such sequences may be obtained by probing cDNA libraries made from or genomic DNA libraries from other mycobacterial species and their isolates, and probing such libraries with probes comprising all or part of SEQ ID. No. 1 as defined above under conditions of medium to high stringency (for example 0.03M sodium chloride and 0.03M sodium citrate at from about 50°C to about 60°C). Homologues of the *mpa* gene in other pathogenic *M. avium* may also be obtained by polymerase chain reaction or other method of amplifying these *mpa* genes using primers derived from SEQ ID No. 1 or the complement thereof.

The invention includes allelic variants and species homologues which may also for example be obtained using degenerate PCR which will use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences.

The primers will contain one or more degenerate positions and will be used at stringency

- 5 conditions lower than those used for cloning sequences with single sequence primers against known sequences.

Alternatively, such polynucleotides may be obtained by site directed mutagenesis of the *mpa* sequence or allelic variants thereof. This may be useful where for example any silent 10 codon changes are required to sequences in order to optimise codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction enzyme recognition sites, or to alter the property or function of the polypeptides encoded by the polynucleotides.

- 15 Such altered property or function will include the addition of amino acid sequences of consensus signal peptides known in the art to effect transport and secretion of the modified polypeptide of the invention. Another altered property will include mutagenesis of a catalytic residue or generation of fusion proteins with another polypeptide. Such fusion proteins may be with an enzyme, with an antibody or with a cytokine or other ligand for a 20 receptor, to target a polypeptide of the invention to a specific cell type *in vitro* or *in vivo* or to enhance immune recognition and reactivity.

The invention further provides double stranded polynucleotides comprising a polynucleotide of the invention and its complement.

25

Polynucleotides or primers of the invention may carry a revealing label. Suitable labels include radioisotopes such as  $^{32}\text{P}$  or  $^{35}\text{S}$ , enzyme labels, or other protein labels such as biotin. Such labels may be added to polynucleotides or primers of the invention and may be detected using by techniques known *per se*.

30

- Polynucleotides or primers of the invention or fragments thereof labelled or unlabelled may be used by a person skilled in the art in nucleic acid-based tests for detecting or sequencing *mpa* and its homologues in samples of body fluids, tissues or excreta from animals and humans and to food and environmental samples e.g. water. Human and animal body fluids
- 5 include sputum, blood, serum, plasma, saliva, milk, urine, csf, semen, faeces and infected discharges. Tissues include intestine, mouth ulcers, skin, lymph nodes, spleen, lung and liver obtained surgically or by a biopsy technique. Animals particularly include commercial livestock such as cattle, sheep, goats, deer, rabbits but wild animals and animals in zoos may also be tested.
- 10 Such tests for detecting generally comprise bringing a biological sample containing DNA or RNA into contact with a probe comprising a polynucleotide or primer of the invention under hybridising conditions and detecting any duplex formed between the probe and nucleic acid in the sample. Such detection may be achieved using techniques such as PCR
- 15 or by immobilising the probe on a solid support, removing nucleic acid in the sample which is not hybridised to the probe, and then detecting nucleic acid which has hybridised to the probe. Alternatively, the sample nucleic acid may be immobilised on a solid support, and the amount of probe bound to such a support can be detected. Suitable assay methods of this any other formats can be found in for example WO89/03891 and WO90/13667.
- 20 Methods for sequencing *mpa* or IS1612 and their homologues include bringing a biological sample containing target DNA or RNA into contact with a probe comprising a polynucleotide or primer of the invention under hybridising conditions and determining the sequence by, for example the Sanger dideoxy chain termination method (see Sambrook *et al.*)
- 25 Such a method generally comprises elongating, in the presence of suitable reagents, the primer by synthesis of a strand are to the target DNA or RNA and selectively terminating the elongation reaction at one or more of an A, C, G or T/U residue; allowing strand elongation and termination reaction to occur; separating out according to size the elongated products to determine the sequence of the nucleotides at which selective
- 30 termination has occurred. Suitable reagents include a DNA polymerase enzyme, the

deoxynucleotides dATP, dCTP, dGTP and dTTP, a buffer and ATP. Dideoxynucleotides are used for selective termination.

Polynucleotides of the invention or fragments thereof labelled or unlabelled may also be  
5 used to identify and characterise different strains of *MAP*, and other *mpa*-containing pathogenic mycobacteria and properties such as drug resistance or susceptibility. The probes of the invention may conveniently be packaged in the form of a test kit in a suitable container for storage and transport. In such kits the probe may be bound to a solid support where the assay format for which the kit is designed requires such binding. The kit may  
10 also contain suitable reagents for treating the sample to be probed, hybridising the probe to nucleic acid in the sample, control reagents, instructions, and the like. The kit also preferably involves methods known in the art such as PCR or LCR.

The use of polynucleotides of the invention in the diagnosis of inflammatory diseases such  
15 as Crohn's disease or sarcoidosis in humans or Johne's disease in animals form preferred aspects of the invention. The polynucleotides may also be used in the prognosis of these diseases. For example, the response of a human or animal subject in response to antibiotic, vaccination or other therapies may be monitored by utilising the diagnostic methods of the invention over the course of a period of treatment and following such treatment.

20 The invention provides probes capable of detecting the presence of polynucleotides of the invention (which can for example be used in the detection or diagnostic methods mentioned herein). Typically such probes are polynucleotide. However, such probes can be non-polynucleotides, such as proteins (for example protein polynucleotides).

25 The use of *mpa* polynucleotides (particularly in the form of probes and primers) of the invention in the above-described methods form a further aspect of the invention, particularly for the detection, diagnosis or prognosis of *MAP* infections.

The present invention also provides polynucleotides encoding the polypeptides of the invention described below. Because such polynucleotides will be useful as sequences for recombinant production of polypeptides of the invention, it is not necessary for them to be selectively hybridisable to the sequence of SEQ ID No. 1 although this will generally be  
5 desirable. Otherwise, such polynucleotides may be labelled, used, and made as described above if desired. Preferably the polynucleotides of the invention encode polypeptides which possess *mpa* activity. *Mpa* polypeptides of the invention are described below.

The present invention also provides the polynucleotide sequence of an IS21-like insertion element hereafter termed IS1612 and related polynucleotide sequences as described in items vi-xi and relevant passages above (hereafter referred to as the IS1612 polynucleotides of the invention). The invention in this aspect also provides a polynucleotide construct comprising the IS1612 polynucleotides of the invention which construct is in a suitable form for transfection into *mpa*-containing bacterial cells,  
15 preferably *mpa*-containing mycobacterial cells originating from an animal or human. The invention further provides a method for attenuating a normally pathogenic mycobacterium whose pathogenicity is mediated in all or in part by the presence or expression of the *mpa* polynucleotides (i-v and relevant passages above) of the invention, which method comprises transfecting into a mycobacterial cell or isolate of said pathogenic  
20 mycobacterium a polynucleotide construct comprising the IS1612 polynucleotides of the invention.

Transfection can be carried out by electroporation or any other method known in the art such that the function of the *mpa* gene is knocked out, resulting in a mutated attenuated  
25 form of these normally pathogenic mycobacteria.

The IS1612 element encodes from SEQ ID No. 4 two polypeptides the first of which in its entirety is new and forms part of the present invention. The entire amino acid sequence of the first polypeptide, termed *istA*, is encoded by nucleotides 98-1678 of SEQ ID No. 4 and

is given as SEQ ID No. 5. The ISI612 polypeptides from the invention are further described below.

### B. Polypeptides.

5

Polypeptides of the invention include polypeptides in substantially isolated form which comprise the sequence set out in SEQ ID No. 2 or SEQ ID No. 5. Polypeptides further include variants of such sequences, including naturally occurring variants and synthetic variants which are substantially homologous to said polypeptides. In this context, 10 substantial homology is regarded as a sequence which has at least 70%, e.g. 80%, 90%, 95% or 98% amino acid homology (identity) over 30 amino acids with the sequence of SEQ ID No. 2.

Polypeptides of the invention also include fragments of the above mentioned full length 15 polypeptides and variants thereof, including in particular any fragments of the sequence set out in SEQ ID No. 2 and the fragment having the sequence of amino acids 1-211 as set out in SEQ ID No. 5. Preferred fragments such as residues 158-211 and 380-444 of SEQ ID No 2, include those which include an epitope and especially in the use of related polypeptides to SEQ ID No. 2 an epitope which is specific to the pathogenicity of the 20 mycobacterial cell from which the polypeptide is derived. Suitable fragments will be at least 8, e.g. 10, 12, 15 or 20 amino acids in size. Polypeptide fragments of *mpa* and allelic and species variants thereof may contain one or more (e.g. 2, 3, 5, or 10) substitutions, deletions or insertions, including conserved substitutions, or modifications of amino acids such as phosphorylation of serine or threonine.

25

Conserved substitutions may be made according to the following table which indicates conservative substitutions, where amino acids on the same block in the second column and preferably in the same line in the third column may be substituted for each other:

5	ALIPHATIC	Non-polar	G A P I L V
		Polar - uncharged	C S T M
			N Q
	Polar - charged	D E K R	
	AROMATIC		H F W Y
	OTHER		N Q D E

- 10 Epitopes may be T-cell epitopes or antibody epitopes determined, for example, by techniques such as peptide scanning techniques as described by Geysen *et al*, Mol. Immunol., 23, 709-715 (1986) as well as other techniques known in the art.

The term "an epitope which is specific to the pathogenicity of the mycobacterial cell" means that the epitope is encoded by a portion of the *mpa* gene, or by the corresponding ORF sequences in other mycobacteria which can be used to distinguish mycobacteria which are pathogenic from related non-pathogenic mycobacteria including non-pathogenic species of *M. avium*. This may be determined using routine methodology. A candidate epitope may be prepared and used to immunise an animal such as a rat or rabbit in order to generate antibodies. The antibodies may then be used to detect the presence of the epitope in pathogenic mycobacteria and to confirm that non-pathogenic mycobacteria do not contain any proteins which react with the epitope. Epitopes may be linear or conformational.

- 25 Polypeptides of the invention may be in a substantially isolated form. It will be understood that the polypeptide may be mixed with carriers or diluents which will not interfere with the intended purpose of the polypeptide and still be regarded as substantially isolated. A polypeptide of the invention may also be in a substantially purified form, in which case it will generally comprise the polypeptide in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the polypeptide in the preparation is a polypeptide of the invention. Polypeptides of the invention may be modified for example by the addition of histidine residues to assist their purification or by the addition of a signal sequence to promote their

secretion from a cell, or a transmembrane sequence to facilitate their immobilisation in a membrane or lipid bilayer.

Thus, polypeptides of the invention include in particular fusion proteins which comprise a 5 polypeptide encoded all or part of the *mpa* gene fused at its N- or C-terminus to a second sequence to provide the desired property or function. Sequences which promote secretion from a cell include, for example the yeast  $\alpha$ -factor signal sequence.

A polypeptide of the invention may be labelled with a revealing label. The revealing label 10 may be any suitable label which allows the polypeptide to be detected. Suitable labels include radioisotopes e.g.  $^{125}\text{I}$ , enzymes, antibodies, polynucleotides and linkers such as biotin. Labelled polypeptides of the invention may be used in diagnostic procedures such as immunoassays in order to determine the amount of a polypeptide of the invention in a sample. Polypeptides or labelled polypeptides of the invention may also be used in 15 serological or cell mediated immune assays for the detection of immune reactivity to said polypeptides in animals and humans using standard protocols.

A polypeptide or labelled polypeptide of the invention or fragment thereof may also be 20 fixed to a solid phase, for example the surface of an immunoassay well or dipstick. Such labelled and/or immobilised polypeptides may be packaged into kits in a suitable container for transport and storage along with suitable reagents, controls, instructions and the like.

Such polypeptides and kits may be used in methods of detection of antibodies or cell 25 mediated immunoreactivity, to the mycobacterial proteins and peptides encoded by the *mpa* gene and its allelic variants and fragments, using immunoassay. Such host antibodies or cell mediated immune reactivity will occur in humans or animals with an immune system which detects and reacts against polypeptides of the invention. The antibodies may be present in a biological sample from humans or animals. The biological sample may be a sample as defined above particularly blood, milk or saliva.

30 Immunoassay methods are well known in the art and will generally comprise:

- (a) providing a polypeptide of the invention comprising an epitope bindable by an antibody against said mycobacterial polypeptide;
- (b) incubating a biological sample with said polypeptide under conditions which allow for the formation of an antibody-antigen complex; and
- 5 (c) determining whether antibody-antigen complex comprising said polypeptide is formed.

Immunoassay methods for cell mediated immune reactivity in animals and humans are also well known in the art (e.g. as described by Weir et al 1994, J.Immunol Methods 176; 93-101) and will generally comprise:

- (a) providing a polypeptide of the invention comprising an epitope bindable by a lymphocyte or macrophage or other cell receptor;
- (b) incubating a cell sample with said polypeptide under conditions which allow for a cellular immune response such as release of cytokines or other mediator to occur; and
- 15 (c) detecting the presence of said cytokine or mediator in the incubate.

Polyptides of the invention may be made by synthetic means (e.g. as described by Geysen *et al.*, 1996) or recombinantly, as described below.

20

Preferred polyptides of the invention include Sequence ID No.2 and particularly residues 158-211 and 380-444 thereof, and amino acids 1-211 of Sequence ID No.5. Fragments as defined above from these regions are particularly preferred. The polyptides and fragments thereof may contain amino acid alterations as defined above.

25

Polyptides of the invention or fragments thereof labelled or unlabelled may also be used to identify and characterise different strains of *MAP*, or other *mpa*-containing pathogenic mycobacteria and properties such as drug resistance or susceptibility.

The use of polypeptides of the invention in the diagnosis of inflammatory diseases such as Crohn's disease or sarcoidosis in humans or Johne's disease in animals form a preferred aspect of the invention. The polypeptides may also be used in the prognosis of these diseases. For example, the response of a human or animal subject to antibiotic or other 5 therapies may be monitored by utilizing the diagnostic methods of the invention over the course of a period of treatment and following such treatment. The use of *mpa* polypeptides of the invention in the above-described methods form a further aspect of the invention, particularly for the detection, diagnosis or prognosis of mycobacterial preferably *MAP* infections.

10

Polypeptides of the invention may also be used in assay methods for identifying candidate chemical compounds which will be useful in inhibiting, binding to or disrupting the function of said polypeptides required for pathogenicity. In general, such assays involve bringing the polypeptide into contact with a candidate inhibitor compound and observing 15 the ability of the compound to disrupt, bind to or interfere with the polypeptide. There are a number of ways in which the assay may be formatted. For example, those polypeptides which have an enzymatic function may be assayed using labelled substrates for the enzyme, and the amount of, or rate of, conversion of the substrate into a product measured, e.g. by chromatography such as HPLC or by a colourimetric assay. Suitable 20 labels include <sup>35</sup>S, <sup>125</sup>I, biotin or enzymes such as horse radish peroxidase.

Candidate chemical compounds which may be used may be natural or synthetic chemical compounds used in drug screening programmes. Extracts of plants which contain several characterised or uncharacterised components may also be used.

25

Alternatively, a polypeptide of the invention may be screened against a panel of peptides, nucleic acids or other chemical functionalities which are generated by combinatorial chemistry. This will allow the definition of chemical entities which bind to polypeptides of the invention. Typically, the polypeptide of the invention will be brought into contact with 30 a panel of compounds from a combinatorial library, with either the panel or the polypeptide

being immobilized on a solid phase, under conditions suitable for the polypeptide to bind to the panel. The solid phase will then be washed under conditions in which only specific interactions between the polypeptide and individual members of the panel are retained, and those specific members may be utilised in further assays or used to design further panels of candidate compounds. Binding of polypeptides of the invention to specific ligands may be identified using an antibody to said polypeptide or a polypeptide bearing a revealing label.

For example, a number of assay methods to define peptide interaction with peptides are known. For example, WO86/00991 describes a method for determining mimotopes which comprises making panels of catamer preparations, for example octamers of amino acids, at which one or more of the positions is defined and the remaining positions are randomly made up of other amino acids, determining which catamer binds to a protein of interest and re-screening the protein of interest against a further panel based on the most reactive catamer in which one or more additional designated positions are systematically varied. This may be repeated throughout a number of cycles and used to build up a sequence of a binding candidate compound of interest.

WO89/03430 describes screening methods which permit the preparation of specific mimotopes which mimic the immunological activity of a desired analyte. These mimotopes are identified by reacting a panel of individual peptides wherein said peptides are of systematically varying hydrophobicity, amphipathic characteristics and charge patterns, using an antibody against an antigen of interest. Thus in the present case antibodies against a polypeptide of the invention may be employed and mimotope peptides from such panels may be identified. We particularly recognise that antibodies, including monoclonal antibodies, to GPL's from pathogenic *MAP* may be identified by their ability to bind to these organisms, but not to mutated forms in which the function of the *mpa* gene has been knocked-out. These antibodies may be used to screen phage-displayed peptide libraries for immunogenic mimics of GPL epitopes, and that such peptide immunogenic mimics may be used in the development of anti-GPL vaccines [Phalipon *et al.* Eur.J.Immunol. 27:2620-2625, 1997].

C. Vectors.

Polynucleotides of the invention can be incorporated into a recombinant replicable vector.

The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a

5 further embodiment, the invention provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells are described below in connection with expression vectors.

10

D. Expression Vectors.

Preferably, a polynucleotide of the invention in a vector is operably linked to a regulatory

sequence which is capable of providing for the expression of the coding sequence by the

15 host cell, i.e. the vector is an expression vector. The term "operably linked" refers to a

juxtaposition wherein the components described are in a relationship permitting them to

function in their intended manner. A regulatory sequence "operably linked" to a coding

sequence is ligated in such a way that expression of the coding sequence is achieved under

condition compatible with the control sequences.

20

The term "regulatory sequences" includes promoters and enhancers and other expression

regulation signals. These may be selected to be compatible with the host cell for which the

expression vector is designed. For example, yeast regulatory sequences include

*S. cerevisiae* GAL4 and ADH promoters, *S. pombe* nmt1 and adh promoters. Mammalian

25 promoters, such as β-actin promoters, may be used. Mammalian promoters also include the

metallothionein promoter which can upregulate expression in response to heavy metals

such as cadmium and is thus an inducible promoter. Tissue-specific promoters, for example

neuronal cell specific may be used. Viral promoters may also be used, for example the

Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the promoter rous

30 sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus

(CMV) IE promoter, herpes simplex virus promoters or adenovirus promoters. All these promoters are readily available in the art.

Such vectors may be transformed into a suitable host cell as described above to provide for expression of a polypeptide of the invention. Thus, in a further aspect the invention provides a process for preparing polypeptides according to the invention which comprises cultivating a host cell transformed or transfected with an expression vector as described above under conditions to provide for expression by the vector of a coding sequence encoding the polypeptides, and recovering the expressed polypeptides.

1.0

The vectors may be for example, plasmid, virus or phage vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell. The vector may also be adapted to be used *in vivo*, for example in a method of gene therapy or a DNA vaccine.

2.0 The use of mammalian host cells is expected to provide for such post-translational modifications (e.g. myristylation, glycosylation, truncation, ligation and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the invention. Such cell culture systems in which polypeptide of the invention are expressed may be used in assay systems to identify 2.5 candidate substances which interfere with or enhance the functions of the polypeptides of the invention in the cell.

A further embodiment of the invention provides host cells transformed or transfected with the vectors for the replication and expression of polynucleotides of the invention. The cells

will be chosen to be compatible with the said vector and may for example be bacterial, yeast, insect or mammalian.

Polynucleotides according to the invention may also be inserted into the vectors described

5 above in an antisense orientation in order to provide for the production of antisense RNA. Antisense RNA or other antisense polynucleotides may also be produced by synthetic means. Such antisense polynucleotides may be used in a method of controlling the levels of *mpa* or its variants or species homologues.

10 E. Antibodies.

The invention also provides monoclonal or polyclonal antibodies to polypeptides of the invention or fragments thereof. Preferred fragments include amino acid residues 158-211 and 380-444 of SEQ ID No. 2. The invention further provides a process for the production

15 of monoclonal or polyclonal antibodies to polypeptides of the invention: Monoclonal antibodies may be prepared by conventional hybridoma technology using the polypeptides of the invention or peptide fragments thereof, as immunogens. Polyclonal antibodies may also be prepared by conventional means which comprise inoculating a host animal, for example a rat or a rabbit, with a polypeptide of the invention or peptide fragment thereof and recovering immune serum. In order that such antibodies may be made, the invention

20 also provides polypeptides of the invention or fragments thereof haptenised to another polypeptide for use as immunogens in animals or humans or admixed with adjuvants.

For the purposes of this invention, the term "antibody", unless specified to the contrary, includes fragments of whole antibodies which retain their binding activity for a tumour target antigen. Such fragments include Fv, F(ab') and F(ab')<sub>2</sub> fragments, as well as single chain antibodies. Furthermore, the antibodies and fragments thereof may be humanised antibodies, e.g. as described in EP-A-239400.

Antibodies may be used in method of detecting polypeptides of the invention present in biological samples by a method which comprises:

30 (a) providing an antibody of the invention;

- (b) incubating a biological sample with said antibody under conditions which allow for the formation of an antibody-antigen complex; and
- (c) determining whether antibody-antigen complex comprising said antibody is formed.

5

Antibodies of the invention may be used to detect *MAP* in a sample, such as a tissue sample or milk, using standard immunohistochemical techniques, which method comprises:

- (a) providing an antibody of the invention;
- 10 (b) treating the tissue section or other sample with lysozyme or process to access the epitope;
- (c) incubating the tissue section or other sample with said antibody under conditions which allow the formation of an antibody-antigen complex; and
- 15 (d) revealing the presence of bound antibody by any one of the standard histochemical methods known in the art.

Antibodies of the invention may be bound to a solid support and/or packaged into kits in a suitable container for storage and transport along with suitable reagents, controls, instructions and the like. Antibodies of the invention may be used in the detection, 20 diagnosis and prognosis of diseases as discussed above in relation to polypeptides of the invention.

#### F. Compositions.

- 25 The present invention also provides compositions comprising a polynucleotide or polypeptide of the invention together with a carrier or diluent. Compositions of the invention also include compositions comprising a nucleic acid, particularly an expression vector, of the invention. Compositions further include those carrying a recombinant virus of the invention. Such compositions include pharmaceutical compositions in which case 30 the carrier or diluent will be pharmaceutically acceptable.

- Pharmaceutically acceptable carriers or diluents include those used in formulations suitable for inhalation as well as oral, parenteral (e.g. intramuscular or intravenous or transcutaneous) administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of
- 5       pharmacy. Such methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both.
- 10      For example, formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostatics and solutes which render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents, and liposomes or other microparticulate systems such as gold
- 15      particles for use in the DNA gun, all of which are designed to target the polynucleotide or the polypeptide of the invention to blood components or one or more organs, or to target cells such as skin cells, dendritic cells, muscle cells, or mucosal M cells of the intestine after oral administration or rectal administration or of the nasal and respiratory mucosa.
- 20      G. Vaccines.
- In another aspect, the invention provides novel vaccines for the prevention and treatment of infections caused by *MAP* and other *mpa*-containing pathogenic mycobacteria in animals and humans. The term "vaccine" as used herein means an agent used to stimulate the
- 25      immune system of a vertebrate, particularly a warm blooded vertebrate including humans, so as to provide protection against future harm by an organism to which the vaccine is directed or to assist in the eradication of an organism in the treatment of established infection. The immune system will be stimulated by the production of cellular immunity and antibodies, desirably neutralizing antibodies, directed to epitopes found on or in a
- 30      pathogenic mycobacterium which expresses the *mpa* gene of the invention. The antibody

- so produced may be any of the immunological classes, such as the immunoglobulins A, D, E, G or M. Vaccines which stimulate the production of IgA are interest since this is the principle immunoglobulin produced by the secretory system of warm-blooded animals, and the production of such antibodies will help prevent infection or colonization of the
- 5 intestinal tract. However an IgM and IgG response will also be desirable for vaccination against systemic infections such as Crohn's disease or Johne's disease.

Vaccines of the invention include naked nucleic acid vaccines such as DNA vaccines. Naked nucleic acid vaccines of the invention include polynucleotides of the invention or  
10 fragments thereof in suitable vectors which may be administered as naked nucleic acid using standard protocols. The preferred nucleic acid vaccines of the invention are DNA vaccines which comprise at least one DNA polynucleotide of the invention incorporated into a plasmid under the control of a strong promoter. Suitable strong promoters are viral promoters such as the Muloney murine leukaemia virus long-terminal repeat (MMLV  
15 LTR), the promoter rouse sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE promoter, herpes simplex virus promoters or adenovirus promoters. The preferred promoter is CMV. The nucleic acid vaccines of the invention made be administered intradermally, subcutaneously, intramuscularly or mucosally by inhalation, ingestion or intra-rectal installation. The nucleic acid vaccine of  
20 the invention may be incorporated into a suitable formulation, absorbed or coated onto particles which are appropriate for ingestion into macrophages or antigen presenting cells. Such particles may include gold particles and may be administered by using a gene gun. The nucleic acid vaccine of the invention may also be carried within another organism such as disabled *salmonella*, *M.Bovis BCG*, *M. Smegmatis* or other mycobacteria,  
25 *Corynebacteria*, or other agents according to established protocol.

Immunising formulations may advantageously include an adjuvant.

When the polynucleotide of the invention is administered as a nucleic acid vaccine, the  
30 amount of nucleic acid administered is typically in the range of from 50-500 µg. Uptake of

- the nucleic acid vaccines of the invention by mammalian cells may be enhanced by several transfection techniques, for example, those including the use of transfection agents. Examples of these agents include cationization (for example calcium phosphate and DEAE-dextran) and lipofectants (for example lipofectam™ and transfectam™). Confirmation of
- 5 successful expression of *mpa* in a bacterium can be achieved by analysing surface sugars for acetylation using high pressure liquid chromatography and other methods known in the art. Typically, the nucleic acid in the vaccine is mixed with the transfection agent to produce a composition. Preferably the nucleic acid vaccine with or without transfection agent is combined with a pharmaceutically acceptable carrier or diluent to produce a
- 10 pharmaceutical composition. Suitable carriers and diluents include isotonic saline solution, for example, phosphate buffered saline. The route of administration and dosages described are intended only a guide since the skilled practitioner will be able to readily determine the optimum route of administration and dosage for any particular patient and condition.
- 15 In addition to the above it is also possible to prepare "live" vaccines containing non-pathogenic microorganisms which express or are capable of causing to be expressed one or more polypeptides of the invention. Non-pathogenic microorganisms may be naturally non-pathogenic microorganisms or attenuated microorganisms which are normally pathogenic. Suitable attenuated microorganisms are known in the art and include
- 20 attenuated bacteria such as attenuated salmonella and attenuated viruses such as vaccinia virus. Transformation of the *mpa* gene or other polynucleotides of the invention into the attenuated microorganism is preferably completed *in vitro*. It is also within the invention to transfet cells of a human or animal to be vaccinated with a polynucleotide of the invention *in vitro* although it is preferred that transfection of a host cell should occur *in vivo* by, for
- 25 example, DNA vaccination as described above.
- It shall also be appreciated that the invention provides a non-pathogenic microorganism or human or animal cell which has on its surface a cell surface component produced by modification of a cell surface substrate with a polypeptide of the invention. Preferably, the
- 30 polypeptide of the invention is expressed within the non-pathogenic microorganism or

human or animal cell upon which the modified cell surface component resides. Thus the invention provides a non-pathogenic microorganism or human or animal cell which has been transformed or transfected with a polynucleotide of the invention which is expressed and results in the presence of the modified cell surface component. It should also be  
5 appreciated that it is within this invention to treat the surface of cells exhibiting the cell surface substrate with preformed polypeptide of the invention to produce cells having the modified cell surface component. It is also within this invention to provide a non-pathogenic microorganism or human or animal cell wherein an identical antigenic determinant to the modified cell surface component is produced on a cell surface using  
10 polypeptides other than *mpa* which fulfil an identical role to *mpa*.

In a further aspect vaccines of the invention which function by expressing *mpa* including, for example, nucleic acid vaccines or live vaccines mentioned above, may be augmented by expressing all of the polypeptides encoded for in the ORFs of the GS region identified  
15 in *MAP* along with *mpa* of the invention. The polypeptides encoded for by the ORFs of GS in *MAP* and the polynucleotide sequence of GS in *MAP* were disclosed and characterised in PCT/GB96/03221. These vaccines may be obtained by transforming the attenuated microorganism mentioned above with a polynucleotide of the invention which comprises the *mpa* gene or previously defined related sequence thereof together with the genes  
20 encoding the polypeptides associated with the GS region in *MAP*. For the naked nucleic acid vaccines transfection of a host cell *in vivo* may be achieved by nucleic acid vaccination using the *mpa* gene or the previously defined related sequence thereof together with the genes encoding the polypeptides associated with the GS element of *MAP* under the control of one or more suitable promoters.

25

Polypeptides of the invention or fragments thereof in substantially isolated form may also be used as vaccines by injection, inhalation, oral administration or by transcutaneous application according to standard protocols. Adjuvants (such as Iscoms or poly(lactide-coglycolide encapsulation), cytokines such as IL-12 and other immunomodulators may be  
30 used for the selective enhancement of the cell mediated or humeral immunological

responses. Vaccination with polynucleotides and/or polypeptides of the invention may be undertaken to increase the susceptibility of pathogenic mycobacteria to antimicrobial agents *in vivo*.

- 5 In instances wherein the polypeptide is correctly configured so as to provide the correct epitope, but is too small to be immunogenic, the polypeptide may be linked to a suitable carrier.

A number of techniques for obtaining such linkage are known in the art, including the  
10 formation of disulphide linkages using N-succinimidyl-3-(2-pyridylthio) propionate (SPDP) and succinimidyl 4-(N-maleimido-methyl)cyclohexane-1-carboxylate (SMCC) obtained from Pierce Company, Rockford, Illinois, (if the peptide lacks a sulphydryl group, this can be provided by addition of a cysteine residue). These reagents create a disulphide linkage between themselves and peptide cysteine residues on one protein and an amide  
15 linkage through the epsilon-amino on a lysine, or other free amino group in the other. A variety of such disulphide/amide-forming agents are known. See, for example, Immun. Rev. (1982) 62:185. Other bifunctional coupling agents form a thioether rather than a disulphide linkage. Many of these thio-ether-forming agents are commercially available and include reactive esters of 6-maleimidocaproic acid, 2-bromoacetic acid, 2-iodoacetic  
20 acid, 4-(N-maleimido-methyl) cyclohexane-1-carboxylic acid, and the like. The carboxyl group can be activated by combining them with succinimide or 1-hydroxyl-2-nitro-4-sulfonic acid, sodium salt. Additional methods of coupling antigens employs the rotavirus/"binding peptide" system described in EP 259,149, the disclosure of which is incorporated herein by reference. The foregoing list is not meant to be exhaustive, and  
25 modifications of the named compounds can clearly be used.

Any carrier may be used which does not itself induce the production of antibodies harmful to the host. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins; polysaccharides, such as latex functionalized Sepharose 7, agarose, cellulose,  
30 cellulose beads and the like; polymeric amino acids, such as polyglutamic acid and

- polylysine, and the like; amino acid copolymers; and inactive virus particles. Especially useful protein carriers are serum albumens, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, tetanus toxoid, and other proteins well known to those skilled in the art. Particularly useful also are controlled release microspheres
- 5 composed of polylactide coglycolide typically 75:25 and typically 50 $\mu$ M in diameter. Polynucleotide and polypeptide vaccines of the invention adsorbed onto such microspheres may be zinc stabilised. Lamella polymers of chitin or chitosan in microparticulate form typically 100 nM may also be employed.
- 10 The immunogenicity of the epitopes may also be enhanced by preparing them in mammalian or yeast systems fused with or assembled with particle-forming proteins such as, for example, that associated with hepatitis B surface antigen. See, e.g., US-A-4,722,840. Constructs wherein the epitope is linked directly to the particle-forming protein coding sequences produce hybrids which are immunogenic with respect to the epitope. In
- 15 addition, all of the vectors prepared include epitopes specific to HBV, having various degrees of immunogenicity, such as, for example, the pre-S peptide. In addition, portions of the particle-forming protein coding sequence may be replaced with codons encoding an epitope of the invention. In this replacement, regions which are not required to mediate the aggregation of the units to form immunogenic particles in yeast or
- 20 mammals can be deleted, thus eliminating additional HBV antigenic sites from competition with the epitope of the invention.

Vaccines may be prepared from one or more immunogenic polypeptides of the invention.

- 25 The polypeptides for use in vaccines may be expressed in various host cells (e.g., bacteria, yeast, insect, or mammalian cells), or alternatively may be isolated from viral preparations or made synthetically.

- 30 The preparation of vaccines which contain an immunogenic polypeptide(s) as active ingredients, is known to one skilled in the art. Typically, such vaccines are prepared as

injectables, or as suitably encapsulated oral preparations and either liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to ingestion or injection may also be prepared. The preparation may also be emulsified, or the protein encapsulated in liposomes. The active immunogenic ingredients are often mixed with

5 excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine. Examples of

10 adjuvants which may be effective include but are not limited to: aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three

15 components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 7 80 emulsion. Adjuvants may also include MF59 or sub-units of Cholera toxin which may be mutated or fragments thereof, or DNA itself which is an adjuvant known in the art. The effectiveness of an adjuvant may be determined by measuring the amount of antibodies directed against an

20 immunogenic polypeptide containing an antigenic sequence resulting from administration of this polypeptide in vaccines which are also comprised of the various adjuvants.

In a further aspect of the invention there is provided an attenuated vaccine comprising a normally pathogenic mycobacteria which harbours an attenuating mutation in the *mpa* gene or one or more of its homologues.

The mycobacteria may be used in the form of a killed bacteria or as a live attenuated vaccine. There are advantages to using a live attenuated vaccine. If the whole live organism is used rather than dead cells or selected cell components which may exhibit modified or denatured antigens, protein antigens in the outer membrane will tend to maintain their

tertiary and quaternary structures and therefore the potential to illicit a good protective long term immunity should be higher.

The term attenuating mutation and the like refers to any genetic lesion in a gene which  
5 renders the gene non-functional. This may be, for example, by deletion of all or part of the gene, point mutation in the coding sequence resulting in a truncated gene product unable to carry out the normal function of the gene, or insertion or interruption of the gene by a nucleotide element which prevents the gene product being coded or causes any gene product generated to be such that it cannot carry out the normal function of the gene.

10

A mutation introduced into an attenuated bacterium of the invention would generally be a non-reverting attenuating mutation. Non-reverting means that for practical purposes the probability of the mutated gene being restored to its normal function is small, for example, less than 1 in  $10^6$  preferably less than 1 in  $10^9$  or more preferably less than 1 in  $10^{12}$ . The  
15 attenuated mycobacteria of the invention may be isolated form. This is usually desirable when the bacteria are to be used for the purposes of vaccination. The term "isolated" means that the bacteria are in a form in which they can be cultured, processed or otherwise used in a form which can be readily identified and in which it is substantially uncontaminated by other bacterial strains, for example, non-attenuated parent strains or unrelated bacterial  
20 strains. The term "isolated bacterium" thus encompasses cultures of a bacterial mutant of the invention, for example, in the form of colonies on a solid medium or in the form of a liquid culture, as well as frozen or dried preparations of the strain.

In a preferred aspect the attenuated mycobacterium is formed by insertion of an insertion  
25 element into the *mpa* gene. The insertion element may consist of a sequence of one or more, preferably ten or more, nucleotides. In preferred attenuated mycobacteria the *mpa* gene is attenuated by insertion of a known insertion element, for example, an IS21-like element, more preferably by insertion of the insertion element IS1612 or an insertion element at least 75% homologous to it. Most preferably the insertion of the IS1612

element or its homologue occurs at the target site 5'-ATGCAC -3' between nucleotides 202 and 209 of the *mpa* sequence. The mycobacteria which is attenuated is preferably *MAP*.

The attenuated mycobacterium may further comprise at least one additional mutation. This

5 may be a mutation in a gene responsible for the production of products essential to bacterial growth which are absent in a human or animal host. For example, mutations to the gene for aspartate semi-aldehyde dehydrogenase (*asd*) have been proposed for the production of attenuated strains of *salmonella*. The *asd* gene is described further in Gene (1993) 129, 123-128. A lesion in the *asd* gene, encoding the enzyme aspartate *B*-

10 semialdehyde dehydrogenase would render the organism auxotrophic for the essential nutrient diaminopelic acid (DAP), which can be provided exogenously during bulk culture of the vaccine strain. Since this compound is an essential constituent of the cell wall for gram-negative and some gram-positive organisms and is absent from mammalian or other vertebrate tissues, mutants would undergo lysis after about three rounds of division in such

15 tissues. Analogous mutations may be made to the attenuated mycobacteria of the invention. Such mutations may also include disabling genes of the GS element including *gsc* and *gsd*.

In addition or in the alternative, the attenuated mycobacteria may carry a *recA* mutation.

20 The *recA* mutation knocks out homologous recombination - the process which is exploited for the construction of the mutations. Once the *recA* mutation has been incorporated the strain will be unable to repair the constructed deletion mutations. Such a mutation will provide attenuated strains in which the possibility of homologous recombination to with DNA from wild-type strains has been minimized. *RecA* genes have been widely studied in the art and their sequences are available. Further modifications may be made for additional

25 safety.

The invention further provides a process for preparing a vaccine composition comprising an attenuated bacterium according to the invention process comprises (a) inoculating a culture vessel containing a nutrient medium suitable for growth of said bacterium; (b)

culturing said bacterium; (c) recovering said bacteria and (d) mixing said bacteria with a pharmaceutically acceptable diluent or carrier.

- The *mpa* gene can also be inactivated by methods known in the art including other  
5 transposon mutagenesis, and allelic exchange.

- Attenuated mycobacterial strains according to the invention may be constructed using recombinant DNA methodology which is known per se. In general, bacterial genes may be mutated by a process of targeted homologous recombination in which a DNA construct  
10 containing a mutated form of the gene is introduced into a host bacterium which it is desired to attenuate. The construct will recombine with the wild-type gene carried by the host and thus the mutated gene may be incorporated into the host genome to provide a bacterium of the present invention which may then be isolated.
- 15 The mutated gene may be obtained by introducing deletions into the gene, e.g. by digesting with a restriction enzyme which cuts the coding sequence twice to excise a portion of the gene and then religating under conditions in which the excised portion is not reintroduced into the cut gene. Alternatively frame shift mutations may be introduced by cutting with a restriction enzyme which leaves overhanging 5' and 3' termini, filling in and/or trimming  
20 back the overhangs, and religating. Similar mutations may be made by site directed mutagenesis. These are only examples of the types of techniques which will readily be at the disposal of those of skill in the art.

- Various assays are available to detect successful recombination. In the case of attenuations  
25 which mutate a target gene necessary for the production of an essential metabolite or catabolite compound, selection may be carried out by screening for bacteria unable to grow in the absence of such a compound. Bacteria may also be screened with antibodies or nucleic acids of the invention to determine the absence of production of a mutated gene product of the invention or to confirm that the genetic lesion introduced - e.g. a deletion -  
30 has been incorporated into the genome of the attenuated strain. In addition, GPL sugars

may be extracted and analysed by high pressure liquid chromatography and other methods such as TLC known in the art.

The concentration of the attenuated strain in the vaccine will be formulated to allow  
5 convenient unit dosage forms to be prepared. Concentrations of from about  $10^4$  to  $10^9$  bacteria per ml will generally be suitable, e.g. from about  $10^5$  to  $10^8$  such as about  $10^6$  per ml. Live attenuated organisms may be administered subcutaneously or intramuscularly at up to  $10^8$  organisms in one or more doses, e.g. from around  $10^5$  to  $10^8$ , e.g. about  $10^6$  or  $10^7$  organisms in a single dose.

10

The vaccines of the invention are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration such as ingestion, rectal installation, or mucosally by inhalation. For suppositories, traditional binders and carriers may include, for  
15 example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1% - 2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of  
20 solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10% - 95% of active ingredient, preferably 25% - 70%. Oral formulations may include enteric coatings so that vaccines are preferentially released in the small intestine or colon.

25 The proteins may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids such as acetic, oxalic, tartaric, maleic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such  
30 as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such

organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccines are administered in a manner compatible with the dosage formulation, and in  
5 such amount as will be prophylactically and/or therapeutically effective. The quantity to be  
administered, which is generally in the range of 500 $\mu$ g to 5mgs of antigen per dose,  
depends on the subject to be treated, capacity of the subject's immune system to synthesize  
antibodies, mode of administration and the degree of protection desired. Precise amounts of  
active ingredient required to be administered may depend on the route of administration or  
10 on judgement of the practitioner and may be peculiar to each subject.

The vaccine may be given in a single dose schedule, or preferably in a multiple dose  
schedule. A multiple dose schedule is one in which a primary course of vaccination may be  
with 1-10 separate doses, followed by other doses given at subsequent time intervals  
15 required to maintain and or reinforce the immune response, for example, at 1-4 months for  
a second dose, and if needed, a subsequent dose(s) after several months. The dosage  
regimen will also, at least in part, be determined by the need of the individual and be  
dependent upon the judgement of the practitioner.

20 The vaccines of the invention may be administered to recipients to treat established  
diseases or in order to protect them against diseases caused by the corresponding wild type  
mycobacteria such as inflammatory diseases such as Crohn's disease or sarcoidoses in  
humans or Johne's disease in animals. The vaccine may be administered by any suitable  
route as described above.

25

The invention is now illustrated by the following Examples which should be construed as  
non-limiting.

Examples**Characterisation of the *mpa* gene.**

- 5 PCR performed on *MAP* DNA using a number of polynucleotide primers derived from the sequence of GS within *M.avium* sub sp. *silvaticum* (PCT/GB96/03221) failed to yield products with *MAP*. Although the first 209 nucleotides of *mpa* are present in *M.avium* subsp.*silvaticum*, the existence of an open reading frame was not apparent because the sequence was truncated in *M.avium* subsp.*silvaticum* by the IS21-like element, now  
10 designated IS1612. Primers including those from within IS1612 that were predicted to produce an amplification product and disclose further DNA sequences from within *MAP*, if this were the same as *M.avium* subsp.*silvaticum*, did not work. The genomic organisation of *MAP* in this region was found to be very different from that of *M.avium* subsp.*silvaticum*. Furthermore, the full extent of the IS21-like element (IS1612) in  
15 *M.avium* subsp.*silvaticum* remained unknown. When further experimentation disclosed the full sequence of IS1612 in *M.avium* subsp.*silvaticum*, primers selected on the basis of this new information again failed to give an amplification product, and reveal further DNA sequence of *MAP*. It became clear that a DNA sequencing strategy for this region of *MAP*, down stream of the GS element known in the art, based upon the DNA sequence  
20 information from *M.avium* subsp.*silvaticum* would not work.

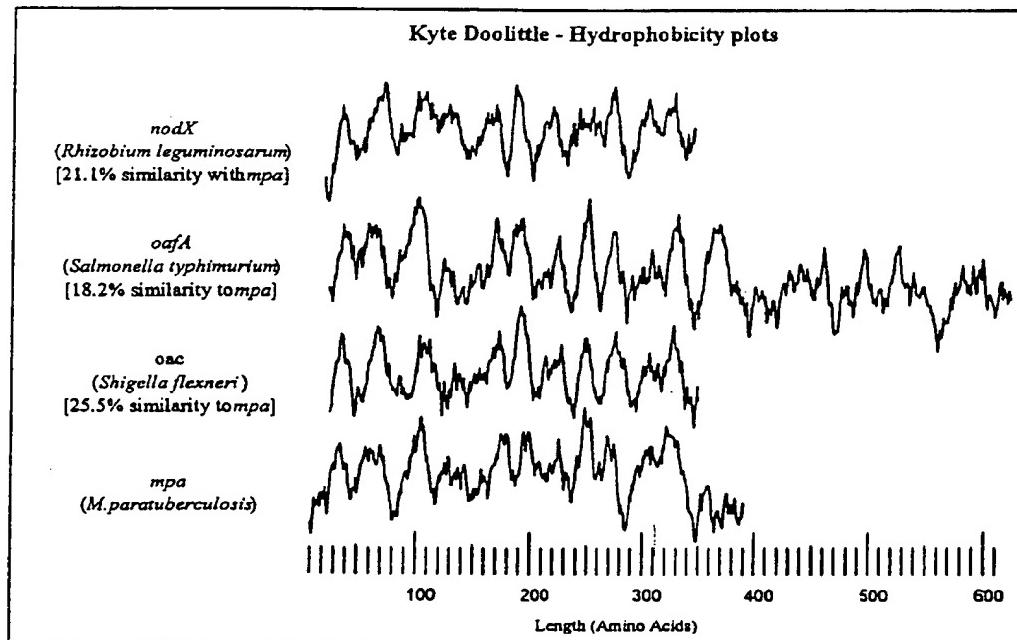
In a new strategy, we used the enzymes *Bam* HI, *SaII*, *Pst*I and *Xho*I and a variety of primers selected from the last known 150bp of DNA sequence in this region of *MAP*, to obtain amplification products from *MAP* DNA using inverse PCR. This involved  
25 recircularisation of genomic digest and subsequent amplification by a set of 'inward' and 'outward' primers. The results were at first difficult to assess because of the production of a number of non-specific amplification products. In several experiments using this strategy however, one band of 280 bp was consistently obtained. This suggested that at least one of the primers in the reaction was specific for further sequence of *MAP*. This 280bp product  
30 was therefore selected from amongst the other amplification products for a further round of

cloning and DNA sequencing. This resulted in our obtaining 250bp of sequence from within the *mpa* gene of *MAP*. Further work revealed that *MAP* did not contain an IS21-like element (IS1612) found in GS in *M.avium* subsp.*silvaticum*. It became clear that the *mpa* gene existed downstream of the GS element in *MAP*. Using inverse PCR the entire 5 sequence of the *mpa* gene (SEQ ID No. 1) and the *mpa* polypeptide which it encodes (SEQ ID No.2) were obtained and verified by sequencing both strands. Comparison of the sequence of the polypeptide in SEQ ID No. 2 with existing amino acid sequences available in the databases, demonstrated homologies which revealed the function of the *mpa* gene product to be an acetyl transferase closely linked with pathogenicity in other organisms.

10

#### **Functional characterisation of *mpa*.**

Sequence data for *mpa* was compared against known sequences using the BLASTN 1.4.11 [24-Nov-97] [Build 24-Nov-97] search facility at NCBI (website: ncbi.nlm.nih.gov/genbank/) on GenBank Flat File release 105.0 (release date 15.02.98). Homologues to *mpa* 15 were found in acetylases from *S.typhimurium*, *S.flexneri* and *R.leguminosarum*. The function of this ORF as an acetylase is strengthened by its very high similarity in hydrophobicity plots of homologues.



In each of these cases the acetylase gene is crucial for determining the host specificity of the organism. Acetylation of lipopolysaccharide O-antigen by the *oac* gene, carried by a strain specific bacteriophage, converts *S.flexneri* to the more virulent 06 serotype (Clark 5 CA, Beltrame J & Manning PA. Gene 107: 43-52. 1991). Critical mutations or deletions in the *oafA* gene, which causes the acetylation of the exported O-antigen lipopolysaccharide in *S.typhimurium*, destroys an epitope crucial for O5 serotype antibody binding and decreases the virulence of this bacteria for host cells (Slauch JM, Lee AA, Mahan MJ & Mekalanos JJ. J.Bacteriol. 178: 5904-5909. 1996). An *mpa* homologue *nodX* is not present 10 in all species of *Rhizobium*, however when present it causes the nod to be acetylated. This specific modification allows the strain to increase the range of hosts that it can stimulate to nodulate. Nod factors consist of polysaccharides that contain glucose, galactose and fucose residues which are methylated and sometimes acetylated. In mycobacteria also, acetylation of GPL's can be important for host recognition. De-acetylation of the terminal fucose of 15 GPL's from *M.avium* MAC serotype 9 abolishes rabbit anti-serotype 9 antibody agglutination indicating that acetylation plays a key role in strain variability amongst bacteria and may be directly attributable as a virulence factor. In *M.avium* serotype 2, transposon knockouts causing specific GPL to alter terminal sugar derivatisation including acetylation, abolishes pathogenicity. Acetylation of rhamnose in GPL's of *Mycobacterium* 20 *smegmatis* induces resistance to mycobacteriophage D4 by inducing conformational changes that destroy the phage attachment site. Thus in *MAP*, the *mpa* gene is responsible for modification of terminal sugar residues in *MAP* GPL's which are critically important in determining cell surface recognition and receptor binding and are important determinants of pathogenicity. *M.avium* subsp.*silvaticum* differs from *MAP* in this important respect 25 because the *mpa* gene in *M.avium* subsp.*silvaticum* is interrupted by the insertion of IS1612. The use of IS1612 using standard transfection methodologies to knock-out *mpa* in *MAP* produces an attenuated mutated strain. The introduction of such specific mutations, including those in the *mpa* gene, is of use in the provision of attenuated vaccine strains of *MAP* to increase the resistance of animals and humans to infections caused by this 30 organism. Transfection of the *mpa* gene from *MAP* into a recipient bacterium particularly

accompanied by the simultaneous transfection of GS will confer on the recipient the property of synthesising cell surface structures which mimic those of *mpa* and thus also serve as a vaccine to increase the resistance of animals and humans to *MAT* infections.

5   **Transformation of another bacterium by *mpa*.**

- Mpa* specific primers are designed with concatenated SacI sites in p12 and a PstI site in p13 at the 3' ends (P12:AGCGAGCTCACGTGACTGAAGCC ; P13:GCTCTGCAG CCGAACACAAACGC). A 1371 bp PCR product is amplified, purified by gel electrophoresis and cleaned with Qiagen gel clean column. The product is then cloned into T-Vector (Promega) and transformed into *E.coli*. The plasmid is grown up, purified and a PstI insert fragment removed and purified as before. This is subcloned into a mycobacterial shuttle vector. This vector contains a mycobacterial ori, *E.coli* ori, hygromycin antibiotic marker and the hsp60 promoter immediately upstream of the insertion locus. The resulting construct is transformed into *E.coli* and purified to 1mg/ml. This vector is then sequenced to check that the PCR step and cloning steps do not introduce errors into the sequence and that the sequence is inserted in the correct orientation in the vector. *M.smegmatis* (strain MC<sup>2</sup>155) is grown into exponential phase, washed twice in 10% glycerol and diluted to OD260:0.5 in TE x1 (Tris 10mM EDTA 0.1mM). 1µg of plasmid is added and the mixture pulsed at 1000Ω:2.5kV:25fD using a BioRad electroporation unit. Cells are recovered in 500µl SOC (2g Tryptone, 0.5g Yeast extract, 1ml 1M NaCl, 0.25ml 1MKCl, 1ml Mg Salts, 1ml 2M Glucose) at 37°C for 3 hours and then plated onto Middlebrooks 7H11 : 45µg/ml Hygromycin selection plates. Transformants are selected and *mpa* presence checked by *mpa* specific P12/P13 PCR.
- 25   Expression of *mpa* is also checked by making total mRNA preps of transformants in exponential growth phase. This is done by pelleting a 4 day 30ml culture (in Middlebrooks 7H11 : 45µg/ml Hygromycin broth) at 3,000 xg for 20 minutes. This is resuspended in 200µl RNase free water and transferred to a ribolyser tube containing silica beads. 500µl of DSA solution (Divolab No.1:9.6ml, 500mM Na Acetate(pH4.0) 24ml, RNase free water 66.4ml) 500µl Acid Phenol (Water saturated phenol with Na Acetate at pH4.0) and

100µl chloroform/isoamyl alcohol (24:1). This is then ribolysed at a speed setting of 6.5 for 45secs. The tubes are then microfuged for 10 minutes at 13,000 xg. Total mRNA is extracted with equal volume of chloroform/isoamyl alcohol and then precipitated at -70°C with isopropanol for 2 hours. This is then microfuged at 13,000 xg for 20 minutes dried  
5 and resuspended in RNAase free water. Samples are treated with DNAase and then cDNA produced using the P12 primer as template with SuperscriptII Reverse Transcriptase (GibcoBRL). PCR using P12/P13 primers is then performed showing bands of the correct size. Controls without DNAase and with RNAase H treatment are performed in parallel. This demonstrates transcribed copies of *mpa* present in *M.smegmatis*. Translation is  
10 revealed by taking exponential growth cultures of transformants and whole cell lysates using 1 minute sonication in 2% SDS-PAGE buffer. These whole cell protein extracts are then electrophoresed on 1% polyacrylamide gels and western blotted onto nylon membranes. These are then hybridised with rabbit raised, *mpa* peptide antisera and developed with anti-rabbit HRP conjugate/ECL peroxide system.

15

#### Deactivation of *mpa* in *MAP* using *IS1612*

General transposon mutagenesis of *MAP* can be achieved using Tn-Mut. Vectors. These vectors contain the insertion sequence, a kanamycin selection marker, shuttle vector origins 20 of replication (including a thermosensitive Myc-ori) and the counterselectable suicide marker *sacB*, and are of proven ability in the mutagenesis of BCG, *M.tuberculosis*, *M.phlei* and *M.smegmatis*. Selected drug resistant transformants may be screened for interruption of the *mpa* gene. However, we have shown that *mpa* in *M.avium* subsp.*silvaticum* is interrupted by the insertion at a specific site of *IS1612* and that *MAP* does not contain  
25 *IS1612* and its *mpa* gene is intact. *IS1612* cloned from a clinical isolate of *M.avium* subsp.*silvaticum* or *IS1612*-containing mycobacterium can be used in a suitable construct for the specific inactivation of *mpa* in *MAP*. Previous studies have shown that *IS21*-like elements resembling *IS1612* can have a selected DNA marker inserted into a region immediately downstream of the *istB* gene without losing transpositional activity. Using 30 primer mutagesesis a unique restriction site is created 4bp downstream of *IS1612*/*istB*. A

hygromycin selection marker is inserted into this site and the resultant transposon substituted into the Tn-Mut vector in place of IS1096. This vector is introduced by electroporation into *MAP* or a pathogenic *M.avium* containing *mpa*, and successful transformants isolated using the selection and counterselection markers and thermotolerant

5 conditions 30°C. Cultures are subbed at intervals into 2% sucrose media and grown at a non-permissive temperature (37°C). The selection marker is co-transferred with the insertion element and selects for transpositional events whilst the temperature suppresses plasmid replication, inducing plasmid leaching and killing plasmid positives via *sacB* expression. DNA extracted from clones is screened using a gene specific primer and an

10 insertion sequence specific primer. Cloned PCR products are sequenced and checked against known target sequence data. Clones are then cultured and RFLP analysis performed using insertion sequence specific probes to determine insertion frequency. Clones with insertions in the *mpa* gene are selected for use as candidate vaccine strains with attenuated pathogenicity.

15

**CLAIMS**

1. A polynucleotide encoding a protein that comprises mycobacterium paratuberculosis acylase (*mpa*), or a fragment or homologue of said protein, said fragment or homologue having *mpa* activity.  
5
2. A polynucleotide selected from:
  - (a) a polynucleotide comprising the entire nucleotide sequence set out in SEQ ID No. 1 or the complement thereof;
  - 10 (b) a polynucleotide comprising a nucleotide sequence capable of hybridising to a fragment of the nucleotide sequence set out in SEQ ID No. 1, the fragment having the nucleotide sequence of nucleotides 210-1335 of SEQ ID No. 1;
  - (c) a polynucleotide comprising a nucleotide sequence capable of hybridising to the complement of a fragment of the nucleotide sequence set out in SEQ ID No. 1, the fragment having the nucleotide sequence of nucleotides 210-1335 of SEQ ID No. 1;
  - 15 (d) a polynucleotide comprising a polynucleotide sequence which is degenerate as a result of the genetic code to the polynucleotide of SEQ ID No. 1 or a polynucleotide of (c); and
  - (e) a polynucleotide having at least 80% homology to the nucleotide sequence of SEQ  
20 ID No. 1.
3. A polynucleotide according to claim 2 which encodes a polypeptide having *mpa* activity.
- 25 4. A polynucleotide according to claim 1 or 3 wherein the *mpa* activity is the acetylation of cell wall components.
5. A polynucleotide probe or primer which comprises a fragment of at least 15 nucleotides of a polynucleotide selected from:
  - (b) a polynucleotide comprising a nucleotide sequence capable of hybridising to a fragment of the nucleotide sequence set out in SEQ ID No. 1, the fragment having  
30

- the nucleotide sequence of nucleotides 210-1335 of SEQ ID No. 1;
- (c) a polynucleotide comprising a nucleotide sequence capable of hybridising to the complement of a fragment of the nucleotide sequence set out in SEQ ID No. 1, the fragment having the nucleotide sequence of nucleotides 210-1335 of SEQ ID No. 1;
- 5 and
- (d') a polynucleotide comprising a polynucleotide sequence which is degenerate as a result of the genetic code to a polynucleotide sequence of (c).
6. A polypeptide in substantially isolated form which is encoded by a polynucleotide of  
10 any one of claims 1 to 4.
7. A polypeptide in substantially isolated form which comprises the sequence set out in SEQ ID No. 2, or a polypeptide substantially homologous thereto which has *mpa* activity, or a fragment of the polypeptide of SEQ ID No. 2 which has *mpa* activity.  
15
8. A polypeptide according to claim 7 which has the sequence set out in SEQ ID No. 2.
9. A polypeptide comprising at least 8 amino acids which is an immunogenic fragment of a polypeptide defined in claim 7 or 8 and which comprises an epitope which is specific  
20 to the pathogenicity of mycobacterial cells.
10. A vector comprising a polynucleotide as defined in any one of claims 1 to 4.
11. An expression vector comprising a polynucleotide as defined in any one of claims 1  
25 to 4, operably linked to regulatory sequences capable of directing expression of said polynucleotide in a host cell.
12. An antibody capable of recognising a polypeptide as defined in any one of claims 6 to  
9.  
30

13. An antibody according to claim 12 which is a monoclonal antibody or a fragment thereof.

14. A method for detecting the presence or absence of a polynucleotide as defined in any 5 one of claims 1 to 4 in a biological sample which method comprises:

- (a) bringing a biological sample containing DNA or RNA into contact with a probe according to claim 5 under hybridising conditions; and
- (b) detecting any duplex formed between the probe and nucleic acid in the sample.

10 15. A method of detecting the presence or absence of a polypeptide as defined in any one of claims 6 to 9 in a biological sample which method comprises:

- (a) incubating the biological sample with an antibody according to claim 12 or 13 under conditions which allow for the formation of an antibody-antigen complex; and
- (b) determining whether antibody-antigen complex comprising said antibody is formed.

16. A method of detecting the presence or absence of antibodies in a biological sample which method comprises:

- (a) incubating a biological sample with a polypeptide according to any one of claims 6 to 9 comprising an epitope under conditions which allow for the formation of an antibody-antigen complex; and
- (b) determining whether an antibody-antigen complex comprising said polypeptide is formed.

25 17. A method of detecting the presence or absence of cell mediated immune reactivity in an animal or human, to a polypeptide according to any one of claims 6 to 9 which method comprises:

- (a) incubating a cell sample with a polypeptide according to any one of claims 6 to 9 comprising an epitope under conditions which allow for a cellular immune response; and
- (b) detecting the presence of said cellular immune response in the incubate.

18. A test kit for detecting the presence or absence of a pathogenic mycobacterium in a sample which comprises a polynucleotide according to any one of claims 1 to 4 or a polypeptide according to any one of claims 6 to 9 or an antibody according to claim 12 or  
5 13.
19. A pharmaceutical composition comprising (i) a polypeptide according to any one of claims 6 to 9 or a polynucleotide according to any one of claims 1 to 4 or an antibody according to claim 12 or 13 and (ii) a suitable carrier or diluent.  
10
20. A polypeptide according to any one of claims 6 to 9 or a polynucleotide according to any one of claims 1 to 4 or an antibody according to claim 12 or 13, for use in the treatment, prevention or diagnosis of a disease caused by a mycobacterium.
- 15 21. A method of treating or preventing a mycobacterial disease in an animal or human caused by mycobacteria which express a polypeptide according to any one of claims 6 to 9, which method comprises administering to the animal or human an effective amount of said polypeptide.
- 20 22. A method of treating or preventing a mycobacterial disease in animals or humans caused by mycobacteria containing the nucleotide sequence of SEQ ID No. 1, which method comprises administering to the animal or human an effective amount of a polynucleotide according to any one of claims 1 to 4 or a vector according to claim 10 or  
11.  
25
23. A method according to claims 21 or 22 wherein the mycobacterial disease is Johne's disease or Crohn's disease.
24. A method according to any one of claims 21 to 23 for increasing the in vivo  
30 susceptibility of mycobacteria to antimicrobial drugs.

25. A vaccine composition comprising (i) a polypeptide according to any one of claims 6 to 9 or a polynucleotide according to any one of claims 1 to 4 or a vector according to claims 10 or 11 together with (ii) a pharmaceutically acceptable carrier or diluent.
- 5 26. A plasmid containing a polynucleotide sequence according to any one of claims 1 to 4 under the control of a promoter.
27. A plasmid according to claim 26 wherein the promoter is a CMV, MMLV, RSV or SV40 promoter.
- 10 28. A nucleic acid vaccine comprising (i) a plasmid as defined in claim 26 or 27 and (ii) a pharmaceutically acceptable carrier or diluent.
29. A vaccine according to claim 28 which further comprises a transfection agent.
- 15 30. A vaccine comprising (i) a polypeptide as defined in any one of claims 6 to 9, optionally linked to a hapten molecule, and (ii) a pharmaceutically acceptable carrier or diluent.
- 20 31. A non-pathogenic microorganism or a cell from a human or animal species prone to infection by *mpa*-containing mycobacteria comprising a component on its surface which has been modified by a polypeptide according to any one of claims 6 to 9.
- 25 32. A non-pathogenic microorganism or a cell from a human or animal species prone to infection by *mpa*-containing mycobacteria which has been transformed or transfected with a nucleic acid construct comprising a polynucleotide as defined in any one of claims 1 to 4 and 26 to 29.
- 30 33. A non-pathogenic microorganism or a cell from a human or animal species prone to infection by *mpa*-containing mycobacteria wherein the nucleic acid construct according to

claim 32, further comprises a polynucleotide which encodes the polypeptides of the GS region of *MAP*.

34. A non-pathogenic microorganism or a cell from a human or animal species prone to  
5 infection by *mpa*-containing mycobacteria according to claim 32 or 33 wherein the gene or genes present in the nucleic acid construct are expressed.

35. A vaccine comprising (i) a non-pathogenic microorganism or a cell from a human or animal species prone to infection by *mpa*-containing mycobacteria according to any one of  
10 claims 31 to 34 and (ii) a pharmaceutically acceptable carrier or diluent.

36. A non-pathogenic microorganism or a cell from a human or animal species prone to infection by *mpa*-containing mycobacteria comprising on its surface an antigenic determinant capable of being produced by the action of a polypeptide as defined in claim 7  
15 or 8 and which is capable of eliciting antibodies which bind the surface of *MAP*.

37. A normally pathogenic mycobacterium or pathogenic isolate thereof, whose pathogenicity is mediated in all or in part by the presence or expression of a polypeptide as defined in any one of claims 6 to 9, which mycobacterium or isolate harbours an  
20 attenuating mutation in the polynucleotide sequence as defined in any one of claims 1 to 4.

38. A vaccine comprising (i) a non-pathogenic microorganism or cell from a human or animal species as defined in claim 36 or a mycobacterium or isolate as defined in claim 37,  
and (ii) a pharmaceutically acceptable carrier or diluent.

25

39. A vaccine according to claim 38 which comprises a mycobacterium or isolate wherein the attenuating mutation in the mycobacterium or isolate is mediated by the insertion of one or more nucleotides.

30 40. A polynucleotide insertion element selected from:  
(a) a polynucleotide comprising the nucleotide sequence set out in SEQ ID Nos. 3 or 4;

- (b) a polynucleotide comprising a nucleotide sequence capable of hybridising to a fragment of the nucleotide sequence set out in SEQ ID No. 3, the fragment having the nucleotide sequence of nucleotides 1856-2543 of SEQ ID No. 3;
  - 5 (c) a polynucleotide comprising a nucleotide sequence capable of hybridising to a fragment of the nucleotide sequence set out in SEQ ID No. 4, the fragment having the nucleotide sequence of nucleotides 1-688 of SEQ ID No. 4;
  - (d) a polynucleotide comprising a polynucleotide sequence which is degenerate as a result of the genetic code to the polynucleotide of SEQ ID No. 4 or a polynucleotide of (b);
  - 10 (e) a polynucleotide having at least 75% homology to the nucleotide sequence of SEQ ID No. 3; and
  - (f) a polynucleotide having at least 75% homology to the nucleotide sequence of SEQ ID No. 4.
- 15 41. A vaccine according to claim 39 wherein the sequence of one or more nucleotides is a sequence as defined in claim 40.
42. A polynucleotide probe or primer which comprises a fragment of at least 15 nucleotides of a polynucleotide selected from:
- 20 (b) a polynucleotide comprising a nucleotide sequence capable of hybridising to a fragment of the nucleotide sequence set out in SEQ ID No. 3, the fragment having the nucleotide sequence of nucleotides 1856-2543 of SEQ ID No. 3;
  - (c) a polynucleotide comprising a nucleotide sequence capable of hybridising to a fragment of the nucleotide sequence set out in SEQ ID No. 4, the fragment having the nucleotide sequence of nucleotides 1-688 of SEQ ID No. 4; and
  - 25 (d') a polynucleotide comprising a polynucleotide sequence which is degenerate as a result of the genetic code to a polynucleotide of (b).
43. A polypeptide in substantially isolated form which is encoded by a polynucleotide of claim 40.

44. A polypeptide comprising at least 8 amino acids which is an immunogenic fragment of the polypeptide defined in claim 43 and which comprises an *istA* epitope.

45. A vector comprising a polynucleotide as defined in claim 40.

5

46. An expression vector comprising a polynucleotide as defined in claim 40, operably linked to regulatory sequences capable of directing expression of said polynucleotide in a host cell.

10 47. A method for preparing a mycobacterium or pathogenic isolate as defined in claim 37 which method comprises transfecting animal or human isolate of an *mpa* containing pathogenic bacterium with a polynucleotide construct comprising a polynucleotide as defined in claim 40.

15 48. The method of claim 47 wherein transfection is effected by electroporation.

49. The method of claim 47 or 48 wherein the polynucleotide sequence as defined in claim 40 has the nucleotide sequence set out in SEQ ID No. 3 or 4.

20

**SEQUENCE LISTINGS****Sequence ID No.1**

5    Total bases = 1,335

1            GTGACTGAAGCCAATGAGTGCAACTCGCGTCGCAGAAAGGTTTCAAGTCGC  
51            GGTTGAGCAAGACACCGCAAGACTACTGGAGTGCCTGCACAAGCGCCCC  
101          AGCTCGCGCTGAAAGCGGATGCAAAGGGGTTCGAAGCTTGAGCAACATG  
10            151          CGAAGGGGAGAACGGCCTATGAGCCTGGACAGGTTTCGACCCGCGCGC  
201          GAATGCACTTAATGCGTGGCGCTTGGTGTGGCGAGCGGGGTATCCTAT  
251          GGCATTGTTCCGCTCACTGGACGTATGCCGTGGCGCCGTCGTCCAG  
301          TTGCTTGGCCTTGGATGCGTTGATGGTTCTTGGGTCTCCGGCTATCT  
351          CATCGTCTCGAGCTGGCTTCGCAACCCGCAATCCGCCAATACCTCACCG  
15            401          CTCGATGTCTCGTATTCTCCGGTCTGTGGATCTGTCATCTTGACG  
451          GCGTTTGTATCGCTCCGATAGGTGTGGCGCCAGGGCGGTTCGGCCGC  
501          GAAACTACTGATGTCCGGCGCTCCGATCGAGTATGTGCTAAAAGACAGTG  
551          CGGTTTGGATGGTTAAGTCGATATCGGTGGCACACCTCGCGATATTCCA  
601          GTTGCAGGGTATTTGGAACGGTTCTGTGGACATTGGTTGGAGGTGCT  
20            651          TTGCTATATCGGCGTAGCAGTATTTGGTATGCTCGACTTCTTAGTCGCC  
701          GTTGGTTCGTTCCAGGGATATTGATCCTGGCGCTGTCGTGGTGGTGTTC  
751          TTGCCGGCATGGGGCGGAATACACCGCATGCCCTCAAATGCTGCGCGATT  
801          CGCTGTGATGTTTCGGCCGGAGCGTTGCTGTATCAATTCCGTAACGTGA  
851          TTCCGGCTCGGTGGTCCTCGTTGCCGTGGCCTCATTATCGTTGTGGTT  
25            901          TCCCTCTGCCGTGCTGCCGGACTACCGGTTGGTGGCGGCCCTCCGATGGC  
951          GTACCTAATCATCGCTTCGGGTCGCTCATCCACAATCAAAGGATGAGGT  
1001         TCCGCACCGATCTATCCTATGGAGTATATATTATGCGTTCCAATTCA  
1051         CAAGTGCTGGTCCTGTGTGGATTGCCGAGATAAATCCAATCGTTCTG  
1101         CGCGATTCTGTCGCAGCTATTTGCCGCTGCCGCGCTCAGTTGGTTCT  
30            1151        TGGTCGAGAACCTGCAGCTGGAAAGAGTCGTCTCCGGCGAAAAAC

1201 AGTTCAATTGCGCTAGCCAATATGGAAGATGGTGGATCAGTCGGCCGCTC  
 1251 AAATGACATTCCCGGAAGGCAGGGCCCCGCTTTATTGGCGAGAAAAGCCGAAG  
 1301 ATCCTCCCGCGCCGAGCCCAGACCAGGGCTTGAA

5

Sequence ID No.2

Total Residues = 444

10 1 VTEANECSASRKVSVAVEQDTARLLECVHKRPQLAESGCKGVRSLSNM  
 51 RRGERPMSLGQVFDPRANALNAWRLVLASGVILWHSFPLTGRMPWAPFVQ  
 101 LLGLGCVDGFFAVSGYLIVSSWLRNPHPAQYFTARCLRILPGLWICLILT  
 151 AFVIAPIGVGAQGGSAAKLLMSGAPIEYVLKDSAVWMVKFDIGGTPRDIP  
 201 VAGIWNGLWTLGWEVLCYIGVAVFGMLGLLSRRWFVPGILILALSWSVF  
 15 251 LPAWGGIHAIASNAARFAVMFSAGALLYQFRNVIPARWSFVAVGLIIVVV  
 301 SSAVLPDYRLVAALPMAYLIIASGSLIHNRMRFRDLSYGVYIYAFPIQ  
 351 QVLVLCGFAEINPIAFC AISVAILPLAALSWFLVEKPALS WKSRLRRKN  
 401 SSIALANMEDGGSVGRSNDIPGRRARF IGEKAEDPPAPS PRPAL

20

Sequence ID No. 3 = IS1612 positive strand.

1 ATGCACTGTCAATGGCCAAGTAGAAGTCCCCGCTGGTGGCCAGCAGAACG  
 51 CCCCCACTCCGCTGGGGTGGTGGCTAATTCTTGGGGCTCCCTTCTTGT  
 25 101 GGTCGGCGTGGCGCATCCGGTAGGACTCGCCGGAGGTGACGACGATGCTG  
 151 GCGTGGTGCAGCAGCCGATCGAGGATGCTGGCGGGTGGTGTGCTCGGG  
 201 CAGGAATCGCCCCATTGTTGAAGGGCCAATGCGAGGCGATGGCCAGGG  
 251 AGCGGCCGCTCGTAGCCGGCAGCCACGAGCCGGAACAAACAGTTGAGTCCC  
 301 GTGTCGTCGAGCGGGGCGAAGCCGATCTCGTCCAAGATGACCAGATCCGC  
 351 GCAGGAGCAGGGTGTGATGATCTTGCCGACGGTGTGCGGCCAGGCCGC

	401	GGTAGAGGACCTCGATCAGGTGGCGCGGTGAAGTAGCGGACTTTGAAT
	451	CCGGCGTGGACGGCAGCGTCCCCGAGCCGATGAGCAGGTGACTTTGCC
	501	CGTACCAAGGTGGGCCAATGACCGCCAGGTTCTGTTGTGCCGAATCCATT
	551	CCAGGCTCGACAGGTAGTCGAACGTGGCTGCGGTGATGACGATCCGGTG
5	601	ACGTCGAACCCGTCGAGGGTCTTGGTACCGGGAAAGGCTGCGGCCTTGAG
	651	ACGGTTGGCGGTGTTGGAGGCATCGCGGGCAGCGATCTCGGCCTAACCA
	701	ACGTCCGCAAGGATCTCCTCCGGTGTCCAGCGTTGCGTCTTGGCAGCTTGC
	751	AACACCTCGGCGGCGTTGCGCGCACCGTGGCCAGCTTAACCGCCGCAG
	801	CGCCCGTCAAGGTCAAGCAGCCAGCGGTGCCGCCAGGACGGTGCCACCG
10	851	GCTTGGCAGCGGTGGTCATGAGGCCGTCCCGTCGGTGGTGTGATCTTGT
	901	AGGCCTCCAACGAGCGGGTCTCGACGGTGGGCAGATCGAGCACGAGTGCG
	951	TCGCCGGCGGGGGGGGGGGTTGTGGGGTGCCGGCGCCGGCCAGGATCGA
	1001	GCGCACGTCGGCAGCGCGAACCGCGAAACCGCAACCGCCGGCGCAGCG
	1051	CGTCAATCAAAGCCTGTTGCCGTGGCGGCCAAGGCCGAGCAGAATG
15	1101	TCGAGTTCGGATTTCACTGCGCTGGTGTGCGATCGCAGCAGCACCGACGAG
	1151	GAAC TGCTCGCTTCGGTCCCAATGCGCAGAATCGTTCTGCTTGGG
	1201	TTTCGGCGAGGACCACGCAGGGTGCAGGGTCTGGGTCCGTCGTAGTGT
	1251	TCATCGAGGATGGACACCTCACCTGGCTGACGAGCTCGTGCCTGGCAC
	1301	GATCACACCGGTGCGAGGTTCAAACAGGATCAGGGGCCATGATGACCA
20	1351	CCACCGCCACGGTGGCACCGACGAGGCCGTGAGGCACCGAGTAACGAGCT
	1401	GAGCCGTAACGGATGCACGAGAGGCCGTGACCTTACGGCGACCGACCC
	1451	CGAGCCGATCGTCGGCCGAGCGAGGGCAGCTCCCTCAAGACGGTGCCT
	1501	CGTCAACCAAGCGATCGTTGGCACGGCGAGATCTCGAGTGGACCGTG
	1551	GCATTGACCTCGGCCACCATAGTTGCCCTGGCGTTGAGGGCACGTAG
25	1601	GTCGACCTGCTCACCGGCTAACCGAGCTTCGGTCAGCAGCGCACCGCAA
	1651	GGTCGTCTGAGCGTAGCCACAGAGGTTCTCCACGATGCCCTCGATTGC
	1701	GGATCCGCACCGTGGCAGAAGTCCGGAACGAAGCCATAGTGGACCGAA
	1751	TCGCACATAATCCGGTGTGGAACACAACATTGGCGACGACACCACCT
	1801	TGAGGCAGCCCATCCGGTGGCCAGGATCTGGCCGAACCCACCGATC
30	1851	GCCTCGAGGGCTTCGGCTATCATCGCCTGCGTGGTCAGGCTTCTCGTC

	1901	GGCGGCGAACCGCTCAAACGCCACCGCGAATAGGCCAGCTCCGCGCATA
	1951	ACACCATCAGCCCCGGTGGCGCTTCGGCCAATCCATCACCAAGATAGTCA
	2001	CCGGGTGACCAGACCGCCGGACGGCGTTGATGCCGGTTAGCGTTGCCA
	2051	CCATACTTCCTGCTCGGCTACCAGGC GGCGGAAGTTACGGGCCAGCCCT
5	2101	GATACCCGGCAGCTCGGGCGATGGCAGCATCCGCTTCGCCGACATTTG
	2151	CCGTGTATTCTCGACTCGGGTGGCGACTAGATCGGTGAACCGTGTGAG
	2201	GTTGCGTGGCCGTGGTTCCCGCGGGGGCGGCCACCGCCTCGGCCGCT
	2251	CGATGACCCGCTTGACCGTCTTGTGCGTACTACCGCACAGCTCGGCCGCG
	2301	CCGCGATACGACCCGACCTGGTGATACGCCAAATGATGTTCATACGCTC
10	2351	CCTTGCAGACTTCAATAGAGCTCCCTGGCGGTGATCAAGTGACAGTTGG
	2401	CGCTATCACCGTCACCGCCCAGGCCCTCAGCTCCGGAAAAGACACGACG
	2451	AGCCCGCTAAGGAGTGGGACTTCTACCTGGCCACCAGTGGGACTTCCT
	2501	ACTGGCCACAGATGGGACTTCTCATGGCCATGGACATGCAC

(Total = 2543bp)

15

Sequence ID No.4 = IS1612 negative strand to SEQ ID No.3.

	1	GTGCATGTCCATGGCCATGAGAAAGTCCCCTGTGGCCAGTAGGAAGT
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	151	TCACTTGATCACCGCCCAGGGAGCTCTATTGAAGTCTGCAAGGGAGCGTA
	201	TGAACATCATTCCGGCGTATCACCAAGGTCGGGTGATCGCGCGCGGCC
	251	GAGCTGTGGTAGTACGCACAAGACGGTCAAGCGGGTATCGAGCGGGC
25	301	CGAGGCCGGTGGCGCGCCCCCGCGGAACCACGCCACGCAACCTCGACG
	351	CGTTCACCGATCTAGTCGCCACCGAGTCGAGAAATCACACGGCAAGATG
	401	TCGGCGAAGCGGATGCTGCCATGCCAGCTGCCGGTATCAGGGCTC
	451	GGCCCGTAACCTCCGCCCTGGTAGCCGAGCAGGAAGTATGGTGGCGCA
	501	ACGCTAACCGGCATCAACGCCGTCCGGCGGTCTGGTCAACCGGTGACTAT
30	551	CTGGTGATGGATTGGCCGAAGCGGCACCGGGCTGATGGTGTATGCGC

	601	GGAGCTGGCCTATCGCGTGGCGTTTGAGCGGTCGCCGCCGACGAGA
	651	AAGCCTCGACCACGCAGGCGATGATAGCCGAAGCCCTCGAGGCGATCGGT
	701	GGGGTTCCGGCCAAGATCCTGGCCGACCGGATGGGCTGCCTCAAAGGTGG
	751	TGTCGTGCCAATGTTGTTCAACACCGGATTATGTGCGATT CGCGT
5	801	CCCACTATGGCTTCGTTCCGGACTTCTGCCACGGTGC GGATCCGCAATCG
	851	AAGGGCATCGTGGAGAACCTCTGTGGCTACGCTCAGGACGACCTGCGGT
	901	GCCGCTGCTGACCGAAGCTGCGTTAGCCGGTGAGCAGGTCACCTACGTG
	951	CCCTCAACGCCAGGCGCAACTATGGTGC GCCGAGGTCAATGCCACGGTC
	1001	CACTCGGAGATCTGCGCCGTGCCAACGATCGCTTGGTTGACGAGCGCAC
10	1051	CGTCTTGAGGGAGCTGCCCTCGCTGCCGCCGACGATCGGCTCGGGTCGG
	1101	TGCGCCGTAAGGTCGACGGCTCTCGTCATCCGTTACGGCTCAGCTCGT
	1151	TACTCGGTGCCTCAGCGGCTCGTCGGTGCCACCGTGGCGGTGGTGGTCGA
	1201	TCATGGCGCCCTGATCCTGTTGGAACCTTGCACCGGTGTGATCGTGGCCG
	1251	AGCACGAGCTCGTCAGCCAGGTGAGGTGTCATCCTCGATGAACACTAC
15	1301	GACGGACCCAGACCCGCACCCCTCGCGTGGTCTCGCCCCGAAAACCCAAGC
	1351	AGAGAAACGATTCTCGCATTGGAACCGAAGCGCAGCAGTTCTCGTCG
	1401	GTGCTGCTGCGATCGAACACCCGACTGAAATCGAACTCGACATTCTG
	1451	CTCGGCCCTGGCGCCGCCACGGCGAACAGGCTTGATTGACCGCTGCG
	1501	CCGGCGGTTGCGTTGCCGGTTCGCCGCTGCCACGTGCGCTGATCC
20	1551	TGGCCGCCGGCGCCGGCACCCCACAACCCCGCCCCGCCGGCACGCACTC
	1601	GTGCTCGATCTGCCACCGTCGAGACCCGCTCGTTGGAGGCCTACAAGAT
	1651	CAACACCAACCGACGGGACGGCTCATGACCACCGCTGCCAAGCCGGTGGC
	1701	ACCGTCCTCGCGGCACCGCTGGCTGCTGACCTTGACCGGGCTGCGGC
	1751	GGTTGAAGCTGGCACGGTGCACCGCAACGCCGCCAGGTGTTCAAGTC
25	1801	GCCAAGACGCAACGCTGGACACCGGAGGAGATCCTCGGGACGTTGGTTGA
	1851	GGCCGAGATCGCTGCCCGCGATGCCCTCAACACCGCCAACCGTCTCAAGG
	1901	CCGCAGCCTTCCCGGTACCAAGACCCCTCGACGGGTTCGACGTACCGGA
	1951	TCGTCGATCACCGCAGCCACGTTGACTACCTGTCAGCCTGGAATGGAT
	2001	TCGGGCACAAACAGAACCTGGCGGTATTGGCCCACCTGGTACGGGAAAA
30	2051	GTCACCTGCTCATCGGCTGCCGGCACGCTGCCGTACGCCGGATTCAA

2101 GTCCGCTACTTCACCGCCGCCGACCTGATCGAGGTCCCTACCGCGGCCT  
 2151 GGCGACAACACCGTCGGCAAGATCATCGACACCCTGCTCCGCGCGGATC  
 2201 TGGTCATCTTGGACGAGATCGGCTTCGCCCCGCTCGACGACACCGGGACT  
 2251 CAACTGTTGTTCCGGCTCGTGGCTGCCGGCTACGAGCGCCGCTCCCTGGC  
 5 2301 CATCGCCTCGCATTGGCCCTTCGAACAATGGGGCGATTCTGCCCAGC  
 2351 ACACCACCGCCGCCAGCATCCTCGATCGGCTGCTGCACCACGCCAGC  
 2401 GTCGTCACCTCCGGCGAGTCCTACCGGATGCGCCACGCCGACCACAAGAA  
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 2501 CTGGCCACCAGCGGGGACTTCTACTTGGCCATTGACAGTGCAT  
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Sequence ID No.5 = Amino acid sequence of polypeptide designated istA  
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15 1 VSFPGAEGLGGDGDSANCHLITAQGALLKSARERMNIISAYHQVGSYRGA  
 51 51 AELCGSTHKTVKRVIERAEAGGAPPREPRPRNLDFTDLVATRVEKSHGK  
 101 101 MSAKRLMPIARAAGYQGSARNFRRLVAEQEVWWRNANRHQRRAVWSPGD  
 151 151 YLVMDWAEAAPGLMVLCAELAYSRWRFERFAADEKASTTQAMIAEALEAI  
 20 201 GGVPAKILADRMGCLKGGVANVVPTPDYVRFASHYGFVPDFCHGADPQ  
 251 251 SKGIVENLCGYAQDDLAVPLLTEAALAGEQVDLRALNAQAQLWCAEVNAT  
 301 301 VHSEICAVPNDRLVDERTVLRELPSLRPTIGSGSVRRKVDGLSCIRYGSA  
 351 351 RYSVPQRLVGATVAVVVDHGALILLEPATGVIVAEHELVSPGEVSILDEH  
 401 401 YDGPRPAPSRGPRPKTQAEKRFCALGTEAQQFLVGAAAIGNTRLKSELDI  
 25 451 451 LLGLGAAHGEQALIDALRRAVAFRRFRAADVRSILAAGAGTPQPRPAGDA  
 501 501 LVLDLPTVETRSLEAYKINTTDGTAS  
 (Total = 526 aa)

Coded by nucleotides 98 - 1678 of seq ID No 4.

# INTERNATIONAL SEARCH REPORT

Int'l. Application No  
PCT/GB 99/00849

A. CLASSIFICATION OF SUBJECT MATTER					
IPC 6	C12N15/55	C12N15/85	C12N9/18	C07K16/40	C12N1/20
	C12Q1/68	C12Q1/44	A61K39/04	A61K48/00	A61K38/46
	//(C12N1/20,C12R1:32)				

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FR 2 682 967 A (PASTEUR INSTITUT ;UNIV MASSEY (FR)) 30 April 1993 (1993-04-30) claims ---	1
A	EP 0 288 306 A (MCFADDEN JOHN JO ;TAYLOR JOHN HERMON (GB)) 26 October 1988 (1988-10-26) claims --- -/-	1

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

### \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

16 July 1999

Date of mailing of the international search report

30/07/1999

Name and mailing address of the ISA

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Authorized officer

Delanghe, L

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 99/00849

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>CHEMICAL ABSTRACTS, vol. 74, no. 5,      1 February 1971 (1971-02-01)      Columbus, Ohio, US;      abstract no. 20614,      KHACHATURYAN, A. A. ET AL: "Formation of      acylases by hydrocarbon-oxidizing      microorganisms"      XP002109388      abstract      &amp; BIOL. ZH. ARM. (1970), 23(7), 40-7 ,</p>	1

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 99/00849

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:  
**Remark:** Although claim(s) 21 - 24 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

#### Remark on Protest

- The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No	
PCT/GB 99/00849	

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
FR 2682967	A 30-04-1993	WO 9308284	A	29-04-1993
		EP 0666917	A	16-08-1995
		JP 7504082	T	11-05-1995
		NZ 244901	A	26-07-1995
EP 0288306	A 26-10-1988	AT 131533	T	15-12-1995
		AU 624574	B	18-06-1992
		AU 1628688	A	02-12-1988
		CA 1340172	A	08-12-1998
		DE 3854767	D	25-01-1996
		DE 3854767	T	05-09-1996
		DK 524289	A	22-12-1989
		EP 0356450	A	07-03-1990
		WO 8808456	A	03-11-1988
		JP 3503837	T	29-08-1991
		US 5225324	A	06-07-1993

## SEQUENCE LISTING

<110> ST GEORGE'S HOSPITAL MEDICAL SCHOOL

<120> DIAGNOSTICS AND VACCINES FOR MYCOBACTERIAL INFECTIONS  
OF ANIMALS AND HUMANS

<130> N74368A DMG IJB DP

<140> PCT/ GB 99/ 00849

<141> 1999-03-18

<150> UK 9806093.2

<151> 1998-03-20

<160> 5

<170> PatentIn Ver. 2.1

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<211> 1335

<212> DNA

<213> Mycobacterium avium

<400> 1

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<211> 444

<212> PRT

<213> Mycobacterium avium

<400> 2

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Pro Gln Leu Ala Ala Glu Ser Gly Cys Lys Gly Val Arg Ser Leu Ser  
35 40 45

Asn Met Arg Arg Gly Glu Arg Pro Met Ser Leu Gly Gln Val Phe Asp  
50 55 60

Pro Arg Ala Asn Ala Leu Asn Ala Trp Arg Leu Val Leu Ala Ser Gly  
65 70 75 80

Val Ile Leu Trp His Ser Phe Pro Leu Thr Gly Arg Met Pro Trp Ala  
85 90 95

Pro Phe Val Gln Leu Leu Gly Leu Gly Cys Val Asp Gly Phe Phe Ala  
100 105 110

Val Ser Gly Tyr Leu Ile Val Ser Ser Trp Leu Arg Asn Pro His Pro  
115 120 125

Ala Gln Tyr Phe Thr Ala Arg Cys Leu Arg Ile Leu Pro Gly Leu Trp  
130 135 140

Ile Cys Leu Ile Leu Thr Ala Phe Val Ile Ala Pro Ile Gly Val Gly  
145 150 155 160

Ala Gln Gly Gly Ser Ala Ala Lys Leu Leu Met Ser Gly Ala Pro Ile  
165 170 175

Glu Tyr Val Leu Lys Asp Ser Ala Val Trp Met Val Lys Phe Asp Ile  
180 185 190

Gly Gly Thr Pro Arg Asp Ile Pro Val Ala Gly Ile Trp Asn Gly Ser  
195 200 205

Leu Trp Thr Leu Gly Trp Glu Val Leu Cys Tyr Ile Gly Val Ala Val  
210 215 220

Phe Gly Met Leu Gly Leu Leu Ser Arg Arg Trp Phe Val Pro Gly Ile  
225 230 235 240

Leu Ile Leu Ala Leu Ser Trp Ser Val Phe Leu Pro Ala Trp Gly Gly  
245 250 255

Ile His Ala Ile Ala Ser Asn Ala Ala Arg Phe Ala Val Met Phe Ser  
260 265 270

Ala Gly Ala Leu Leu Tyr Gln Phe Arg Asn Val Ile Pro Ala Arg Trp

275	280	285
Ser Phe Val Ala Val Gly Leu Ile Ile Val Val Val Ser Ser Ala Val		
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Leu Pro Asp Tyr Arg Leu Val Ala Ala Leu Pro Met Ala Tyr Leu Ile		
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Ile Ala Ser Gly Ser Leu Ile His Asn Gln Arg Met Arg Phe Arg Thr		
325	330	335
Asp Leu Ser Tyr Gly Val Tyr Ile Tyr Ala Phe Pro Ile Gln Gln Val		
340	345	350
Leu Val Leu Cys Gly Phe Ala Glu Ile Asn Pro Ile Ala Phe Cys Ala		
355	360	365
Ile Ser Val Ala Ala Ile Leu Pro Leu Ala Ala Leu Ser Trp Phe Leu		
370	375	380
Val Glu Lys Pro Ala Leu Ser Trp Lys Ser Arg Leu Arg Arg Lys Asn		
385	390	395
Ser Ser Ile Ala Leu Ala Asn Met Glu Asp Gly Gly Ser Val Gly Arg		
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Ser Asn Asp Ile Pro Gly Arg Arg Ala Arg Phe Ile Gly Glu Lys Ala		
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Glu Asp Pro Pro Ala Pro Ser Pro Arg Pro Ala Leu		
435	440	

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 <212> DNA  
 <213> Mycobacterium avium

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<211> 2543

<212> DNA

<213> Mycobacterium avium

<400> 4

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<211> 526

<212> PRT

<213> Mycobacterium avium

<400> 5

Val Ser Phe Pro Gly Ala Glu Gly Leu Gly Gly Asp Gly Asp Ser Ala

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Asn Cys His Leu Ile Thr Ala Gln Gly Ala Leu Leu Lys Ser Ala Arg

20	25	30
----	----	----

Glu Arg Met Asn Ile Ile Ser Ala Tyr His Gln Val Gly Ser Tyr Arg

35	40	45
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Gly Ala Ala Glu Leu Cys Gly Ser Thr His Lys Thr Val Lys Arg Val

50	55	60
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Ile Glu Arg Ala Glu Ala Gly Ala Pro Pro Arg Glu Pro Arg Pro

65	70	75	80
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Arg Asn Leu Asp Ala Phe Thr Asp Leu Val Ala Thr Arg Val Glu Lys

85	90	95
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Ser His Gly Lys Met Ser Ala Lys Arg Met Leu Pro Ile Ala Arg Ala

100	105	110
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Ala Gly Tyr Gln Gly Ser Ala Arg Asn Phe Arg Arg Leu Val Ala Glu  
115 120 125

Gln Glu Val Trp Trp Arg Asn Ala Asn Arg His Gln Arg Arg Pro Ala  
130 135 140

Val Trp Ser Pro Gly Asp Tyr Leu Val Met Asp Trp Ala Glu Ala Ala  
145 150 155 160

Pro Gly Leu Met Val Leu Cys Ala Glu Leu Ala Tyr Ser Arg Trp Arg  
165 170 175

Phe Glu Arg Phe Ala Ala Asp Glu Lys Ala Ser Thr Thr Gln Ala Met  
180 185 190

Ile Ala Glu Ala Leu Glu Ala Ile Gly Gly Val Pro Ala Lys Ile Leu  
195 200 205

Ala Asp Arg Met Gly Cys Leu Lys Gly Gly Val Val Ala Asn Val Val  
210 215 220

Val Pro Thr Pro Asp Tyr Val Arg Phe Ala Ser His Tyr Gly Phe Val  
225 230 235 240

Pro Asp Phe Cys His Gly Ala Asp Pro Gln Ser Lys Gly Ile Val Glu  
245 250 255

Asn Leu Cys Gly Tyr Ala Gln Asp Asp Leu Ala Val Pro Leu Leu Thr  
260 265 270

Glu Ala Ala Leu Ala Gly Glu Gln Val Asp Leu Arg Ala Leu Asn Ala  
275 280 285

Gln Ala Gln Leu Trp Cys Ala Glu Val Asn Ala Thr Val His Ser Glu  
290 295 300

Ile Cys Ala Val Pro Asn Asp Arg Leu Val Asp Glu Arg Thr Val Leu  
305 310 315 320

Arg Glu Leu Pro Ser Leu Arg Pro Thr Ile Gly Ser Gly Ser Val Arg  
325 330 335

Arg Lys Val Asp Gly Leu Ser Cys Ile Arg Tyr Gly Ser Ala Arg Tyr  
340 345 350

Ser Val Pro Gln Arg Leu Val Gly Ala Thr Val Ala Val Val Asp  
355 360 365

His Gly Ala Leu Ile Leu Leu Glu Pro Ala Thr Gly Val Ile Val Ala  
370 375 380

Glu His Glu Leu Val Ser Pro Gly Glu Val Ser Ile Leu Asp Glu His  
385 390 395 400

Tyr Asp Gly Pro Arg Pro Ala Pro Ser Arg Gly Pro Arg Pro Lys Thr  
405 410 415

Gln Ala Glu Lys Arg Phe Cys Ala Leu Gly Thr Glu Ala Gln Gln Phe  
420 425 430

Leu Val Gly Ala Ala Ala Ile Gly Asn Thr Arg Leu Lys Ser Glu Leu  
435 440 445

Asp Ile Leu Leu Gly Leu Gly Ala Ala His Gly Glu Gln Ala Leu Ile  
450 455 460

Asp Ala Leu Arg Arg Ala Val Ala Phe Arg Arg Phe Arg Ala Ala Asp  
465 470 475 480

Val Arg Ser Ile Leu Ala Ala Gly Ala Gly Thr Pro Gln Pro Arg Pro  
485 490 495

Ala Gly Asp Ala Leu Val Leu Asp Leu Pro Thr Val Glu Thr Arg Ser  
500 505 510

Leu Glu Ala Tyr Lys Ile Asn Thr Thr Asp Gly Thr Ala Ser  
515 520 525